ABSTRACT

Title of Document: MOLECULAR SYSTEMATICS OF NIGHTJARS AND NIGHTHAWKS (CAPRIMULGIDAE)

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Caprimulgidae, a cosmopolitan family of nocturnal or crepuscular insectivorous birds, comprises the subfamilies Caprimulginae (nightjars) and Chordeilinae (nighthawks). A phylogeny was reconstructed using cytochrome *b*, c-*myc* and growth hormone DNA sequences. Likelihood, parsimony and Bayesian analyses identify four major phylogenetic groups, three New World and one Old World. One New World clade consists of whip-poor-wills and relatives; a second consists of two traditional nighthawk genera, *Chordeiles* and *Podager*; a third consists of the remaining Neotropical taxa. *C. enarratus*, a Madagascan endemic, branches before these clades, and has no close relatives among the species sampled. The subfamilies are not monophyletic, suggesting the morphological specializations characterizing "nighthawks" evolved multiple times. *Eurostopodus* forms the earliest branches of the tree and may be paraphyletic. *Caprimulgus* is polyphyletic with

respect to many other genera in the family, which are often defined by distinct plumage traits that may reflect sexual selection.

MOLECULAR SYSTEMATICS OF NIGHTJARS AND NIGHTHAWKS (CAPRIMULGIDAE)

By

Kin-Lan Han

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Advisory Committee:
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Professor Charles Mitter, Co-advisor
Professor Richard Highton

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Introduction

While birds are among the best studied classes of organisms, many questions remain about their basic biology. Nightjars and nighthawks (Caprimulgidae) are a case in point. Because most of their activity occurs in the dark of night, little is known about their behavior or ecology. However, recent research has begun to increase our knowledge of the breeding and foraging biology of these secretive birds (Aragones et al. 1999; Sierro et al. 2001; Jetz et al. 2003; Grand and Cushman 2003; Wichmann 2004; Lane et al. 2004a; Lane et al. 2004b). Better understanding of the evolutionary relationships of Caprimulgidae is needed to provide a comparative framework for interpreting studies on the behavior and ecology of these enigmatic birds.

The Caprimulgidae are a cosmopolitan family of nocturnal or crepuscular insectivorous birds found in a variety of habitats (Cleere 1999). All caprimulgids are cryptically colored in shades of brown and gray. During the day, they generally roost horizontally on the ground, although they can occasionally be found on branches in trees or on rocks. All caprimulgids are aerial foragers. Although they have small bills, they have very wide gapes that are thought to open both horizontally and vertically (Cleere 1998). Presumably, this trait helps them catch flying prey.

Early attempts to describe nightjar relationships were limited to the available morphological and behavioral data (e.g. Sclater 1866a; Sclater 1866b; Beddard 1886; Davis 1962; Ingels 2001; Cleere 2002; Whitney et al. 2003). However, due to their cryptic coloration, conserved appearance, and poorly known behavior, traditional phylogenetic studies based on morphology and behavior have been unsatisfactory in determining the evolutionary history of caprimulgids. This is not unexpected in a group

like Caprimulgidae, since morphological and behavioral characters may be under uniform selection for crypticity and thus, can be misleading due to convergent or parallel evolution. Consequently, relationships within and among nightjars remain contentious (see Systematic Background).

Advances in molecular methods provide a potent alternative to morphological and behavioral phylogenetic analyses. Molecular genetic research, beginning in the 1980s, has begun to unravel some of the complexity of caprimulgid relationships (Sibley and Ahlquist 1990; Mariaux and Braun 1996; Barrowclough et al. 2006). These results indicate a larger degree of genetic diversity present in caprimulgids than would otherwise be expected, given their conserved external morphology. The purposes of this study are to develop a robust phylogeny of Caprimulgidae, test the monophyly of genera and subfamilies within the family and begin the interpretation of character evolution.

Systematic Background

The Caprimulgidae are one of five major lineages within the order Caprimulgiformes. Until the nineteenth century, all Caprimulgiformes were classified into one family and assigned to a single genus *Caprimulgus* (Cleere 1999). Beginning in the nineteenth century, enough morphological differences were recognized to separate species into different genera and families (reviewed by Sibley and Ahlquist 1990; Cleere 1999). Eventually, five families were recognized, Steatornithidae (oilbird), Podargidae (frogmouths), Caprimulgidae (nightjars and nighthawks), Aegothelidae (owlet-nightjars), and Nyctibiidae (potoos). Appendix A gives a more detailed synopsis of caprimulgiform systematics.

While ornithologists generally accepted these five families as being each others' closest relatives, the morphological and genetic diversity was remarked upon, and their relationship to other birds continued to be debated (Sibley and Ahlquist 1990). Recently, monophyly of the order has been questioned. Both morphological and molecular studies have found that the order may not be monophyletic because considerable evidence now indicates that Aegothelidae is sister to Apodiformes (swifts and hummingbirds) (Livezey and Zusi 2001; Mayr 2002; Cracraft et al. 2004; Barrowclough et al. 2006; Braun and Huddleston unpublished). However, an expanded clade of Caprimulgiformes with swifts and hummingbirds nested within it does appear to be a monophyletic group (S. Hackett et al., unpublished data from the Early Bird consortium).

Caprimulgidae is the largest of the five caprimulgiform families, including about 89-90 species (Cleere 1998; Cleere 1999; Holyoak 2001). Two subfamilies are traditionally recognized; Caprimulginae (nightjars) and Chordeilinae (nighthawks), based on morphological and behavioral characters (Peters 1940; Cleere 1998; Cleere 1999; Holyoak 2001). Caprimulginae are found throughout the world and have a schizognathous palate[1] (Cleere 1998; Cleere 1999; Holyoak 2001). Most Caprimulginae have long rictal bristles around the gape and tend to be sit-and-wait predators, sallying after flying insects from perches on the ground or from open limbs. In contrast, the Chordeilinae are only found in the New World and have a desmognathous palate[2] (Cleere 1998). They generally lack rictal bristles and have longer, narrower wings. Chordeilinae

[1] Refers to cleft palates; maxillo-pallatines are separate from each other or vomers in mid-line (Schodde and Mason 1980; Holyoak 2001).
[2] Refers to uncleft palates; broad maxillo-palatines that join each other or the vomers in the midline (Schodde and Mason 1980; Holyoak 2001).

also tend to be more aerial feeders, hunting by hawking on the wing rather than sallying from a perch.

Although as many as 51 genera have been named in the past (Cleere 1999), about 15 genera are currently considered valid (Figure 1). Four genera are traditionally placed within Chordeilinae, *Chordeiles, Lurocalis, Nyctiprogne* and *Podager* (Peters 1940; Cleere 1998; Cleere 1999). The other eleven genera, *Eurostopodus, Nyctidromus, Phalaenoptilus, Siphonorhis, Nyctiphrynus, Caprimulgus, Macrodipteryx, Hydropsalis, Uropsalis, Macropsalis* and *Eleothreptus* are placed within Caprimulginae (Peters 1940; Cleere 1998; Cleere 1999). However, Holyoak (2001) places *Podager* in Caprimulginae

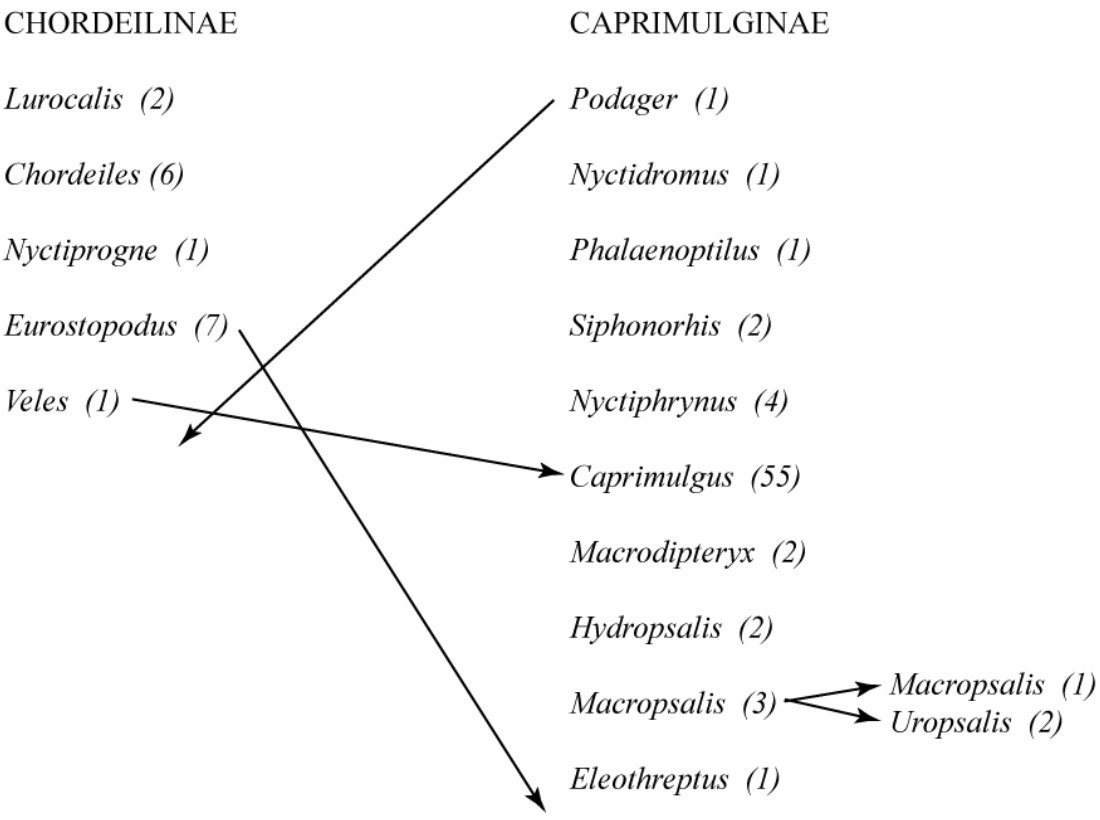

Figure 1: List of genera and number of species.
Nomenclature follows Holyoak (2001). Numbers in parentheses indicates number of species in each genus. Arrows indicate differences from the traditional taxonomy followed by Cleere (1998).

because it has rictal bristles, which are lacking in other chordeilines. Holyoak also places *Eurostopodus* within Chordeilinae based on some morphological affinities with other Chordeilinae. Among these are a lack of rictal bristles, square tails, and narrow, pointed wings (Schodde and Mason 1980; Holyoak 2001). Furthermore, Holyoak recognizes the monotypic Afrotropical genus *Veles* and places it in Chordeilinae, along with *Eurostopodus*. *Veles* had been subsumed within *Caprimulgus*, but a recent review (Cleere 2001) noted several distinctive morphological characters that suggest it may be a valid genus. It has usually been treated as a caprimulgine (Peters 1940; Cleere 2001). To add further confusion to the taxonomy, Holyoak (2001) lumps *Macropsalis* into *Uropsalis* while Cleere (1998) follows Peters (1940) and continues to treat them as distinct genera.

Most caprimulgid species are placed within the genus *Caprimulgus* based on similar external appearance, making it one of the largest of all avian genera (55 species according to Holyoak, 57 according to Cleere). However, DNA-DNA hybridization studies (Sibley and Ahlquist 1990) found large genetic divergences between some purported congeners. In fact, *C. europaeus* had a distance of $\Delta T_{50}H = 7.8$ from *C. vociferus*, a distance found at the subfamily level in other taxa (Sibley and Ahlquist 1990). Examination of the proposed phylogeny according to DNA-DNA hybridization data indicates that the genus *Caprimulgus* may be polyphyletic (Figure 2). This result is further supported by DNA sequencing studies (Cracraft et al. 2004; Barrowclough et al. 2006; Braun and Huddleston unpublished) and suggests revisions are needed in generic allocations within Caprimulgidae. However, taxon sampling within these studies has been too limited to offer suggestions on how to revise the genera. Sibley and

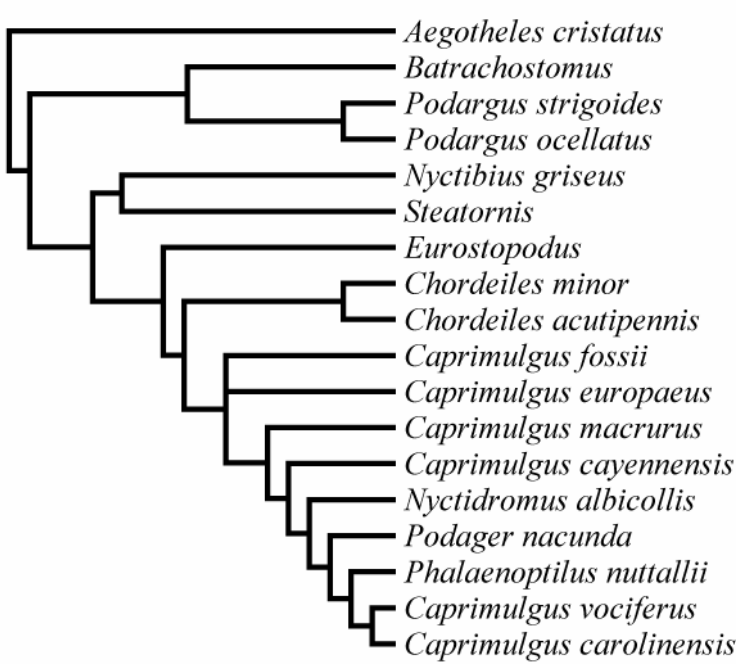

Figure 2: Inferred phylogeny based on DNA-DNA hybridization data (Sibley and Ahlquist 1990).

Ahlquist (1990) suggested that the genus *Antrostomus* was available for the two New World *Caprimulgus*, *C. vociferus* and *C. carolinensis*, but their study only included six species of *Caprimulgus*, leaving the position of others unresolved.

Davis (1962) attempted to describe relationships among North American *Caprimulgus* using acoustic evidence, arguing that species recognition of nocturnal birds would be heavily dependent on voice. In fact, the common names of various North American caprimulgids are based on the bird's song, suggesting vocalizations may be distinct enough to be useful characters for phylogenetic analysis. Based on these studies, Davis (1962) placed *C. cayennensis* and *C. maculicaudus* into the genus *Antiurus*. He also concluded there are two groups within the genus *Antrostomus*:

(1) four species in two subgroups, *rufus* plus *badius* and *salvini* plus *carolinensis*;

(2) three species in two subgroups, *vociferus* and *saturatus* plus *arizonae*.

C. v. arizonae is usually recognized as a subspecies of *C. vociferus* (Cleere 1998; Cleere 1999; Holyoak 2001). However, some studies suggest the two forms may represent separate species (American Ornithologists' Union 1998). Davis (1962) grouped *C. v. arizonae* closer to *C. saturatus* than to *C. vociferus*, suggesting it may be distinct enough to warrant elevation to full species. He left *C. ridgwayi* in *Caprimulgus* with the caveat that more sampling would be needed to further subdivide this genus. With further taxon sampling, Davis (1978) placed *C. ridgwayi* in a genus *Setopagis* along with *C. parvulus*.

Many of the other currently recognized caprimulgid genera are comprised of only one or a few species (Figure 1). These genera are often recognized based on elaborate morphological characters such as elongated tail or wing feathers. Such characters are likely sexually selected and do not necessarily reflect evolutionary history. They may have evolved independently or they may be recently derived autapomorphic characters. For example, the genus *Macrodipteryx* consists of two species of Afrotropical nightjars, *M. longipennis* and *M. vexillarius.* Breeding males are characterized by elongated second innermost primary wing feathers, which are used for sexual display-flights during the breeding season (Cleere 1998; Holyoak 2001). Males are thought to attract females by displaying at leks, suggesting they are polygynous. It is very likely that the elongated feathers evolved relatively rapidly in response to sexual selection. However, differences in plumage, wing shape and structure of the second primaries suggest the two species

may not be closely related at all. In fact, *M. vexillarius* had at one time been placed into its own genus, *Semeïophorus* (Peters 1940; Cleere 1998; Cleere 1999).

The genus *Macropsalis* typically consists of one species, *M. creagra* (also known as *M. forcipata*) (Peters 1940; Cleere 1998; Cleere 1999; Holyoak 2001), although Holyoak synonymizes this genus with *Uropsalis* (Figure 1), which consists of two additional species, *U. segmentata* and *U. lyra*. All three of these species are Neotropical. Males are characterized by having elongated outermost tail feathers. However, the structure of this tail feather differs between the two genera. This difference, when combined with enlargement of the three outermost primaries found only in males of *Macropsalis* (Holyoak 2001), accounts for the distinction between the two genera. It is likely these elongated tail feathers are sexually selected. *U. lyra* is known to display at communal leks (Holyoak 2001) although little is known about whether this is also true in the other two species.

In the past, the genus *Eurostopodus* has been treated as a synonym of *Caprimulgus* (Schodde and Mason 1980). Morphologically, species in *Eurostopodus* tend to be larger and darker in color and lack long rictal bristles. Two species, *E. macrotis* and *E. temminckii*, have "ear tufts" and have previously been recognized in a separate genus, *Lyncornis*. Traditionally, these birds are placed within Caprimulginae although, as previously mentioned, Holyoak (2001) places them with Chordeilinae (Figure 1). However, placing *Eurostopodus* with Chordeilinae groups Old World taxa with what is otherwise strictly a New World group. Holyoak does this primarily based on the lack of rictal bristles, but describes other similarities such as narrow wings and square tails (Schodde and Mason 1980; Holyoak 2001). Presumably, these traits evolved

independently and *Eurostopodus* spp. are distinct nightjars that may be of ancient origin, based on their large genetic divergences.

DNA-DNA hybridization studies (Figure 2) indicate that the genus *Eurostopodus* may be sufficiently different ($\Delta T_{50}H = 12.3$) from the rest of Caprimulginae to warrant placing them into a separate family, Eurostopodidae (Sibley and Ahlquist 1990). Furthermore, studies by Mariaux and Braun (1996), using mitochondrial cytochrome-*b* (cyt*b*), indicated that the two species of *Eurostopodus* sequenced, *E. papuensis* and *E. mystacalis*, always separated from the Caprimulgidae (Figure 3). In fact, they were more distinct from the Caprimulginae than was *Chordeiles*. These results were consistent with Sibley and Ahlquist's recognition of a family, Eurostopodidae. Subsequent molecular studies (Braun and Huddleston unpublished) using cyt*b* and nuclear c-*myc* demonstrate *Eurostopodus* is monophyletic with other caprimulgids, and represents an early radiation of the group. However, these studies indicate the genus *Eurostopodus* itself may not be monophyletic (See Figure 4). *E. macrotis* appears paraphyletic to other *Eurostopodus*, suggesting the genus *Lyncornis* may still be valid.

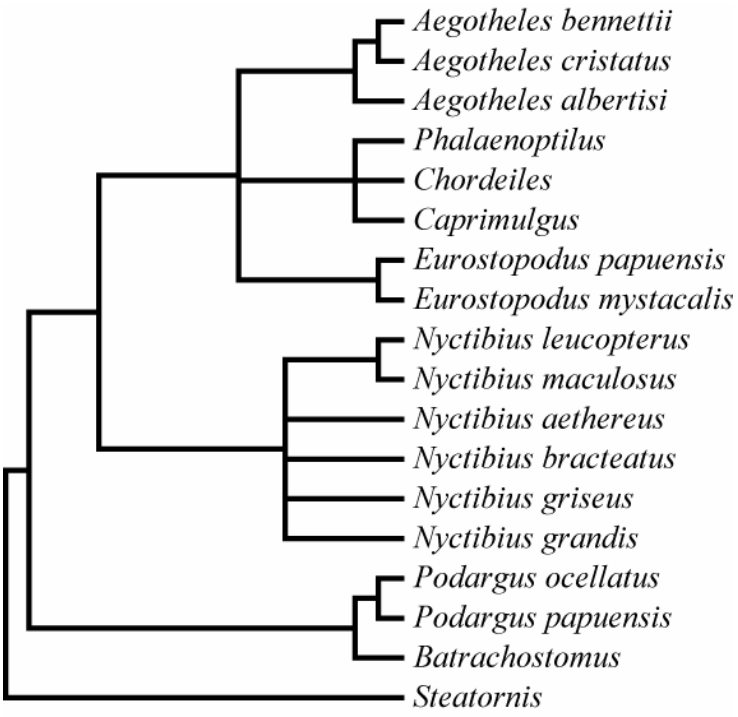

Figure 3: Inferred phylogeny using cyt*b* (Mariaux and Braun 1996)

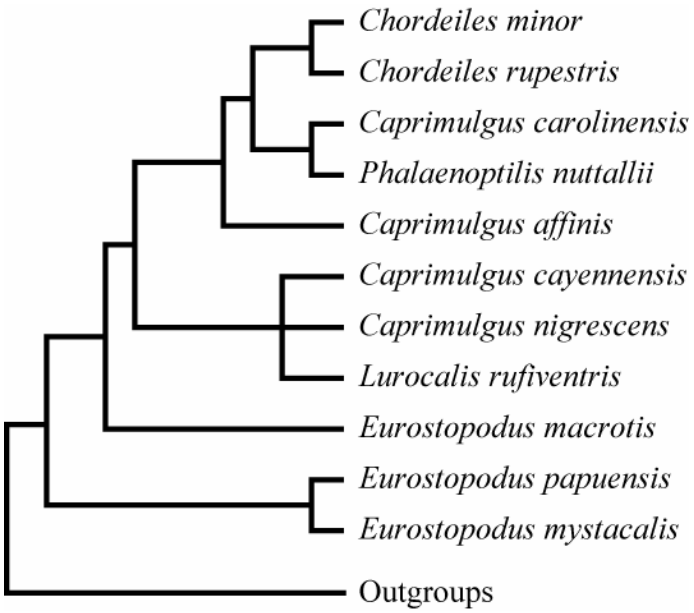

Figure 4: Inferred phylogeny of Caprimulgidae using cyt*b* and c-*myc* (Braun and Huddleston unpublished)

Objectives

In this study, I wished to test two principal hypotheses regarding caprimulgid evolution:

(1) **Are the genera *Caprimulgus* (55-57 species) and *Eurostopodus* (7 species) monophyletic?** Testing monophyly of *Caprimulgus* and *Eurostopodus* will determine whether they are recent and rapidly radiating clades or old and morphologically conserved composite groups. Monophyly would suggest the morphological and behavioral similarities are due to a recent and rapid radiation. The alternative hypothesis, that the genera are not monophyletic, could suggest these birds have maintained a successful lifestyle and body plan while morphologically divergent forms arose from within the group through adaptation and/or sexual selection.

(2) **Are the two traditional subfamilies, Chordeilinae and Caprimulginae, monophyletic?** If both subfamilies are monophyletic, then aerial hawking and sallying, and the morphological adaptations associated with each foraging mode, may have evolved once. If neither subfamily is monophyletic, then one or both of these suites of behavioral and morphological adaptations must have arisen multiple times through convergent evolution.

I tested these hypotheses by constructing a robust phylogeny of Caprimulgidae using molecular sequence data. For this study, I assembled tissue samples from >60% of caprimulgid taxa and 14 of 15 currently recognized genera. I collected DNA sequence data from one mitochondrial (cyt*b*) and two nuclear genes (cellular myelocytomatosis proto-oncogene [c-*myc*] and growth hormone [GH]). Cyt*b* is a rapidly evolving gene that

is best for resolving diversification at the species level to subfamily and possibly family level (Moore and DeFilippis 1997). C-*myc* is a well-studied proto-oncogene that is slowly evolving and more useful for studying deep divergences (Graybeal 1994; Ericson et al. 2000). GH was developed as a probe of phylogeny for Early Bird, a large-scale collaborative project to determine higher level relationships among birds, in which our laboratory is involved. Based on data collected from Early Bird, the evolutionary rate of GH was determined to be intermediate between cyt*b* and c-*myc* for Caprimulgiformes. Cyt*b* and c-*myc* were chosen to correspond with previous studies done in this lab (Braun and Huddleston unpublished). GH was chosen to complement rates of divergence of the other two genes and hopefully provide resolution at various depths of the tree. Phylogenetic analyses were conducted using maximum likelihood, parsimony and Bayesian methods to provide a diverse analytical framework for interpretation of the data.

Materials and Methods

Taxon Sampling

Taxonomic nomenclature follows Holyoak (2001) as it is the most recent treatment of Caprimulgiformes available. Frozen tissue samples were obtained from the museum tissue collections listed in Table 1. I attempted to include one sample of every named species available, and included well marked subspecies on a case by case basis. Sixty-seven specimens of Caprimulgidae were included in the study representing 58 of 89 species and 14 of the 15 genera recognized by Holyoak (2001). Six additional taxa from the other four caprimulgiform families were included as outgroups (Table 1).

Laboratory Methods

DNA Extraction

Total genomic DNA was extracted from frozen tissues using proteinase K digestion, phenol:chloroform extraction and ethanol precipitation (Sambrook et al. 1989; Mariaux and Braun 1996). Phase-Lock gel (Eppendorf) was used during phenol:chloroform extraction to separate the organic and aqueous phases. (Refer to Appendix B for a more detailed protocol). Genomic DNA was visualized on a 1.5% agarose gel to check for chain length and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Working DNA stocks were diluted to a final concentration of 10 ng/μL.

Table 1: Tissue samples used. (Nomenclature follows Holyoak (2001))

SPECIES	TISSUE NO.	LOCATION
OUTGROUP		
Aegotheles insignis	UKAN 5081	Papua New Guinea
Batrachostomus septimus	CMNH B499	Philippines
Podargus strigoides	LSUM B8654	Zoo/Captive
Steatornis caripensis	LSUM B7474	Peru
Nyctibius bracteatus	LSUM B4509	Peru
Nyctibius grandis	LSUM B15415	Bolivia
CAPRIMULGIDAE		
Chordeilinae		
Chordeiles acutipennis 1	USNM B4378	Guyana
Chordeiles acutipennis 2	UKAN 9308	El Salvador
Chordeiles acutipennis 3	UKAN 9367	El Salvador
Chordeiles minor	USNM B7387	Cayman Islands
Chordeiles pusillus	USNM B12993	Guyana
Chordeiles rupestris	ANSP T2755	Ecuador
Lurocalis rufiventris	ANSP 4467	Ecuador
Lurocalis semitorquatus semitorquatus	USNM B5244	Guyana
L. s. nattereri	UKAN 277	Paraguay
Nyctiprogne leucopyga	UKAN 3144	Paraguay
Eurostopodus argus	MVIC C718	Australia
Eurostopodus macrotis	USNM B3732	Philippines
Eurostopodus mystacalis	MVIC JWC129	Australia
Eurostopodus papuensis	MVIC E660	Papua New Guinea
Caprimulginae		
Caprimulgus aegyptius	LSUM B46332	Kuwait
Caprimulgus affinis	FMNH 358300	Philippines
Caprimulgus anthonyi	ANSP 4580	Ecuador
Caprimulgus batesi	USNM B9899	Gabon
Caprimulgus carolinensis	USNM B16552	United States
Caprimulgus cayennensis	USNM B11295	Guyana
Caprimulgus clarus	UMMZ T2159	
Caprimulgus climacurus	FMNH 396431	Ghana
Caprimulgus enarratus	FMNH 431158	Madagascar
Caprimulgus europaeus	LSUM B23375	South Africa
Caprimulgus fossii	ZMUC 115493	Tanzania
Caprimulgus indicus	UWBM 47117	Russia
Caprimulgus longirostris	LSUM B32361	Peru
Caprimulgus macrurus 1	USNM B4000	Papua New Guinea

UKAN=University of Kansas Natural History Museum; CMNH=Cincinnati Museum of Natural History; LSUM=Louisiana State University Museum of Natural Science; USNM=U.S. National Museum of Natural History; ANSP=Academy of Natural Sciences; MVIC=Museum Victoria; FMNH=Field Museum of Natural History; UMMZ=University of Michigan Museum of Zoology; ZMUC=Zoological Museum University of Copenhagen; UWBM= Burke Museum of Natural History and Culture; BARR=Marjorie Barrick Museum of Natural History; CONACYT=Consejo Nacional de Ciencia y Tecnología; OMVP=El Museo de Zoología de la Facultad de Ciencias de la Universidad Nacional Autónoma de Mexico (UNAM)

Table 1 cont'd

SPECIES	TISSUE NO.	LOCATION
Caprimulgus macrurus 2	USNM B5657	Myanmar
Caprimulgus maculicaudus	UKAN 5488	Guyana
Caprimulgus madagascariensis	FMNH 436420	Madagascar
Caprimulgus manillensis 1	USNM B6090	Philippines
Caprimulgus manillensis 2	USNM B3673	Philippines
Caprimulgus nigrescens	USNM B4478	Guyana
Caprimulgus nigriscapularis	FMNH 346199	Uganda
Caprimulgus parvulus 1	UKAN 106	Paraguay
Caprimulgus parvulus 2	USNM B5879	Argentina
Caprimulgus pectoralis	UWBM 71315	South Africa
Caprimulgus poliocephalus	BARR 11252	Malawi
Caprimulgus ridgwayi 1	CONACYT 415	Mexico
Caprimulgus ridgwayi 2	CONACYT 852	Mexico
Caprimulgus ruficollis	UMMZ T2507	Gambia
Caprimulgus rufigena	BARR 7950	South Africa
Caprimulgus rufus	USNM B4420	Guyana
Caprimulgus salvini	CONACYT 328	Mexico
Caprimulgus saturatus	LSUM B28251	Panama
Caprimulgus v. vociferus	UKAN 2457	USA
Caprimulgus v. arizonae 1	BARR 12900	Mexico
Caprimulgus v. arizonae 2	OMVP 406	Mexico
Caprimulgus whitelyi 1	USNM B19022	Guyana
Caprimulgus whitelyi 2	USNM B19106	Guyana
Eleothreptus anomalus	UKAN 3275	Paraguay
Hydropsalis torquata	BARR 6500	Argentina
Hydropsalis climacocerca	USNM B10337	Guyana
Macrodipteryx longipennis	UMMZ T2505	Gambia
Macropsalis lyra	ANSP 5033	Ecuador
Macropsalis segmentata	ANSP 4962	Ecuador
Nyctidromus albicollis	USNM B324	Panama
Nyctiphrynus mcleodii	FMNH 5830	Mexico
Nyctiphrynus ocellatus	LSUM B12445	Bolivia
Nyctiphrynus yucatanicus	UKAN 2110	Mexico
Phalaenoptilus nutallii	USNM B84	United States
Podager nacunda	USNM B2768	Argentina
Nyctiphrynus rosenbergi 1	ANSP B2002	Ecuador
Nyctiphrynus rosenbergi 2	ANSP B2003	Ecuador
Siphonorhis brewsteri	UKAN 8149	Dominican Republic

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Table 1 cont'd

SPECIES	TISSUE NO.	LOCATION
Caprimulgus sp.[◊]	ZMUC 115717	Uganda

[◊] Unidentified species from ZMUC. No voucher available. Possible species are: *C. tristigma, C. natelensis, C. ruwenzorii,* or *C. fraenatus*

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DNA Amplification and Sequencing

DNA was amplified via polymerase chain reaction (PCR) and sequenced for three gene regions: cyt*b*, *c*-myc, and GH. Primers used for PCR amplification and sequencing are listed in Table 2. All PCRs were performed on a MJ Research Tetrad thermocycler. PCR products were visualized in a 1.5% agarose gel.

Cyt*b*

PCR for cyt*b* was performed in 50 µL reactions. Final concentrations were 1x *Taq* DNA Polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.2 mM each dNTP, and 0.2 µM each of primers L14764 and H16060, 0.05 U/µL *Taq* DNA polymerase (Promega) and 0.40 ng/µL template DNA. PCR was performed under the following cycling conditions: initial denaturation at 95°C for five min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for one min, with a final extension at 72°C for 10 min. Cyt*b* PCR products were separated from primers and excess nucleotides by precipitation using polyethylene glycol (PEG) precipitation (Refer to Appendix B for details).

Table 2: Primers used for PCR amplification and sequencing

Gene	Primer	Sequence 5' to 3'	Reference
Cyt*b*	L14764	TGTTACAAAAAAATAGGMCCMGAAGG	Sorenson et al. 1999
Cyt*b*	L15323	CCATGAGGACAAATATCATTCTGAGGTGC	Mariaux and Braun 1996
Cyt*b*	L15749	GCCATCCTACGCTCAATCCC	Braun and Huddleston unpublished
Cyt*b*	H15295	TGATATTTGTCCTCATGG	Braun and Huddleston unpublished
Cyt*b*	H15730	GGGATTGAGCGTAGGATGGC	Braun and Huddleston unpublished
Cyt*b*	H16060	TTTGGYTTACAAGACCAATG	Braun and Huddleston unpublished
c-*myc*	MYC-F-01	TAATTAAGGGCAGCTTGAGTC	Harshman et al. 2003
c-*myc*	MYC-F-02	TGAGTCTGGGAGCTTTATTG	Harshman et al. 2003
c-*myc*	MYC-F-03	AGAAGAAGAACAAGAGGAAG	Harshman et al. 2003
c-*myc*	MYC-F-05	CACAAACTYGAGCAGCTAAG	Harshman et al. 2003
c-*myc*	MYC-R-04	GGCTTACTGTGCTCTTCT	Harshman et al. 2003
c-*myc*	MYC-R-06	TTAGCTGCTCAAGTTTGTG	Harshman et al. 2003
c-*myc*	MYC-R-47	CTATAAAGACTTTATTAAAGGTATTTACAT	This study
GH	GH-F874	CCTTCCCWGCCATGCCCCTTTCCAACC	Yuri et al. unpublished
GH	GH-R1925	TCCCTTCTTCCAGGTCCTTTART	Yuri et al. unpublished
GH	GH-F897	TGTTTGCCAACGCTGTGCTGAGG	Yuri et al. unpublished
GH	GH-R1477	TACCGATTTCTGCTGGGCATCATCCTTC	Yuri et al. unpublished
GH	GH-INT2-F-04	CTCTRARARCAGTGGGAGATGGC	Yuri et al. unpublished
GH	GH-INT2-R-04	GCCATCTCCCACTGYTYTYAGAG	Yuri et al. unpublished
GH	GH-CAP-F-01	GTGAGAGGAAGACTTTTAGG	This study
GH	GH-CAP-R-01	CCTAAAAGTCTTCCTCTCAC	This study
GH	GH-CAP-F-02	GATGAGGAAAGGCTGAGGG	This study
GH	GH-CAP-R-02	CCCTCAGCCTTTCCTCATC	This study

_C_-myc

Initial amplification of c-*myc* was performed in 10 µL reactions. Final concentrations were 1x PCR Buffer (supplied with TaKaRa HS), 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 µg/µL bovine serum albumin (BSA), 0.2 µM each of primers MYC-F-01 and MYC-R-47, 0.05 U/µL *Taq* DNA polymerase (TaKaRa HS), and 0.5 ng/µL template DNA. PCR was performed under the following cycling conditions: initial denaturation at 95°C for five min, followed by 40 cycles of 95°C for 30 sec, 53°C for 30 sec and 72°C for one min, with a final extension at 72°C for 10 min. Sometimes, initial amplifications were weak or produced multiple bands and had to be re-amplified using a 5' nested primer to increase specificity. PCR products were diluted 1:10 and 1 µL of the dilution was re-amplified in 50 µL reactions. Final concentrations were 1x PCR Buffer (GeneChoice) 1.7 mM $MgCl_2$, 0.2 mM each dNTP and 0.2 µM each primers MYC-F-02 and MYC-R-47, to which was added 0.05 U/µL *Taq* DNA polymerase (GeneChoice). Re-amplifications were performed under the following cycling conditions: 95°C for five min followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for one min with a final extension at 72°C for 10 min. Final products were cleaned using PEG precipitation (Appendix B).

GH

Initial amplification of GH was performed in 25 µL reactions. Final concentrations were 1x PCR Buffer (GeneChoice), 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µg/µL BSA, 0.1 mM tetramethylammonium chloride (TMAC), 0.2 µM each primers F874 and R1925, 0.4 U/µL *Taq* DNA polymerase (GeneChoice), and 0.4 ng/µL template DNA. PCR was performed under the following cycling conditions: initial denaturation

at 94°C for three min followed by 10 cycles of 94°C for 30 sec, 70-61°C for 30 sec, decreasing annealing temperature by 1°C per cycle, and 72°C for one min, then 30 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for one min with a final extension at 72°C for 10 min. In most cases, initial amplifications produced multiple bands. PCR products were diluted 1:10 and 1 μL of the dilution was re-amplified using nested primers in 50 μL reactions. Final concentrations were 1x PCR Buffer (GeneChoice), 1.5 mM $MgCl_2$, 1.7 mM each dNTP, 0.1 mM TMAC and 0.25 μM each primers F897 and R1477, and 0.025 U/μL *Taq* DNA polymerase (GeneChoice). Re-amplifications were performed under the following cycling conditions: 94°C for three min followed by 5 cycles of 94°C for 30 sec, 66-62°C for 30 sec, reducing annealing temperature by 1°C per cycle, and 72°C for 45 sec, then 25 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 45 sec, with a final extension at 72°C for 10 min. Final products were cleaned using either PEG precipitation or gel purified using the Qiaquick gel purification kit (Qiagen).

Sequencing

Purified PCR products were cycle sequenced on both strands with the primers listed in Table 2. (Refer to Appendix B for reaction conditions). Excess dye terminators were removed from cycle sequencing reactions using Sephadex G-50 (Sigma) filtration spin columns. Sequences were run on an ABI 3100 capillary DNA sequencer (Applied Biosystems).

Cloning

In some cases, nuclear PCR products had to be cloned due to sequence length heterozygosity. PCR products were cloned using a "TOPO TA for Sequencing" cloning

kit (Invitrogen) to obtain a clean sequence for one or more of the alleles (Refer to Appendix B for more details). Two colonies from each PCR product were isolated and grown overnight in LB liquid culture. Plasmids were purified using a FastPlasmid Mini kit (Eppendorf). The resulting products were sequenced using primers listed in Table 2.

Data Analysis

Sequence assembly and alignment

Sequences were edited and assembled into contiguous fragments using Sequencher 4.5 (GeneCodes). Each taxon was checked for double stranded sequence reads for each gene. Single nucleotide polymorphisms from heterozygotes were coded using standard ambiguity codes. Sequences from cloned fragments were included after removal of vector sequences. In cases where a base from the cloned sequence differed from the direct sequence (due to *Taq* polymerase error), the direct sequence was accepted as correct.

Sequences were initially aligned automatically using ClustalX (Thompson et al. 1997) with default parameters for gap opening and extension costs. Alignments were further improved manually using Se-Al (Rambaut 1996).

Base frequencies for each gene and gene partition were examined using the chi-square test in PAUP*4.0b10 (Swofford 2002) to determine if there was significant base compositional heterogeneity among taxa.

Phylogenetic Analyses

Individual genes

Maximum parsimony analysis (MP) was performed on each individual gene using

PAUP*. A heuristic search of 1000 random taxon addition search replicates and TBR branch swapping was performed with parsimony as the optimality criterion and all characters equally weighted. To test the robustness of the trees, non-parametric bootstrap analyses were run on each gene. For cyt*b*, 1000 bootstrap pseudo-replicates were performed with 100 random sequence additions per pseudo-replicate. However, initial analyses of the nuclear genes found large numbers of equally parsimonious trees and it was necessary to limit the number of trees saved in the bootstrap analyses. One hundred bootstrap pseudo-replicates were performed with 20 random additions per pseudo-replicate. A limit of 100 trees (NCHUCK=100 CHUCKSCORE=1) was placed on the number of trees retained for swapping in each random addition replicate.

A maximum likelihood analysis (ML) was also performed on each gene. Modeltest 3.7 (Posada and Crandall 1998) was used to evaluate models of sequence evolution and select model parameters. A neighbor-joining tree initially was obtained via PAUP* using Jukes-Cantor distances. Parameter values were calculated on this tree for 56 nested models of sequence evolution. The models were evaluated in Modeltest 3.7 using the Akaike Information Criterion. The best model and parameter values were input into a heuristic tree search of 10 random addition replicates and TBR branch swapping using ML as the optimality criterion. Model estimation was then repeated on the resulting tree. This process was repeated until tree topology and model parameter values converged in a successive-approximations approach (Swofford et al. 1996; Sullivan et al. 2005). Models and parameter values for each gene are shown in Table 3. Subsequently, a heuristic search was performed with 10 random sequence additions and TBR branch swapping, using likelihood as the optimality criterion to obtain the final tree. Confidence

Table 3: Parameters used in maximum-likelihood analyses

GENE	MODEL	BASES				SHAPE PARAMETER (α)	PROPORTION OF INVARIABLE SITES (I)
		A	C	G	T		
Cyt*b*	GTR[a] + I[d] + Γ	0.3231	0.4466	0.0648	0.1655	0.6642	0.4612
c-*myc*	TVM[b] + I + Γ	0.3159	0.2053	0.2147	0.2641	0.7604	0.4849
GH	TVMef[c] + Γ	0.2500	0.2500	0.2500	0.2500	0.7038	0.0000
c-*myc* + cyt*b*	GTR + I + Γ	0.3011	0.3591	0.1650	0.1748	0.5947	0.5278
Combined	GTR + I + Γ	0.2731	0.2952	0.2049	0.2268	0.4582	0.3714
Nuclear (c-*myc* + GH)	TVM + I + Γ	0.2727	0.2319	0.2408	0.2546	0.7279	0.3039

[a]GTR=General-time-reversible model; [b]TVM=Transversional model; [c]TVMef=Transversional model with equal base frequencies; [d]I=proportion of invariable sites;

Table 3: cont'd

GENE	RATES					
	A↔C	A↔G	A↔T	C↔G	C↔T	G↔T
Cyt*b*	0.5385	15.1977	1.4056	0.4116	21.0715	1.0000
C-*myc*	0.6586	4.9011	0.2631	0.8169	4.9011	1.0000
GH	1.1449	3.9682	0.5907	1.0019	3.9682	1.0000
c-*myc* + cyt*b*	0.8752	5.1878	1.2045	0.2365	18.6091	1.0000
Combined	1.0681	4.1678	0.7715	0.4450	9.6335	1.0000
nuclear (c-*myc* + GH)	0.9749	4.3743	0.4630	0.9669	4.3743	1.0000

in the hypothesized tree was estimated using 100 bootstrap replicates with one random sequence addition per pseudo-replicate.

A Bayesian analysis (MB) was performed using MrBayes v3.1 (Huelsenbeck and Ronquist 2001). Four MCMC chains were run simultaneously for 10,000,000 generations and sampled every 500 generations. Data collected from the first 1,000,000 generations (2000 trees) were discarded as burn-in. A general-time-reversible model with invariable sites and a gamma distribution (GTR + I + Γ) was used for analysis. For cyt*b*, the analysis was performed with the data partitioned by first, second, and third base positions. For c-*myc* the analysis was performed with three data partitions, intron, exon and UTR. The data was partitioned by coding and noncoding sequences for the GH analysis.

Combined Analyses

A partition homogeneity test or ILD (incongruence length difference) test (Farris et al. 1995) was performed using PAUP* to determine if there was significant conflict between gene partitions.

Pairwise gene combinations

C-*myc* and cyt*b* were analyzed together. For parsimony analysis, 1000 random addition replicates and TBR branch swapping were performed with parsimony as the optimality criterion. To test the robustness of the tree, 1000 bootstrap pseudo-replicates were performed with 100 random sequence additions per pseudo-replicate.

A ML analysis was also performed using PAUP*. An initial starting tree was obtained using GARLI (Zwickl 2006) which uses a genetic algorithm and a GTR + I + Γ model of evolution. Modeltest was then used to select a model and parameter values in

the successive-approximations approach described earlier. Further trees were analyzed using PAUP*. For model and parameter values used, refer to Table 3. A ML heuristic search was performed with 10 random sequence additions and TBR branch swapping using likelihood as the optimality criterion. Confidence in the hypothesized tree was estimated using 100 bootstrap replicates with one random sequence addition per bootstrap replicate.

A Bayesian analysis was performed with MrBayes using a GTR + I + Γ model. Four MCMC chains were run simultaneously for 10,000,000 generations and sampled every 500 generations. Data collected from the first 1,000,000 generations (2000 trees) was discarded as burn-in. The dataset was analyzed with two partitions, one for each gene.

All genes

MP and ML analyses were performed on the combined dataset using PAUP* and the methods previously described. For the ML analysis, a general-time-reversible model was used, with invariable sites and a gamma distribution (GTR + I + Γ) (Table 3).

A MB analysis was performed using four MCMC chains run simultaneously for 10,000,000 generations and sampled every 500 generations. A GTR + I + Γ model was used for analysis. The dataset was analyzed with three partitions, one for each gene. Data from the first 1,000,000 generations (2000 trees) was discarded as burn-in.

Constrained Trees

Constrained tree searches were run on each of the individual gene datasets, as well as the combined and pairwise gene datasets. Trees were constrained for monophyly of each of the subfamilies under the Holyoak classification as well as the more traditional

classification (Peters 1940; Cleere 1998; Cleere 1999). Additional constrained tree searches were run with the two genera, *Caprimulgus* and *Eurostopodus*, as monophyletic. A ML analysis was performed using PAUP* and the best fit model previously described with model parameters optimized using Modeltest in a successive approximations approach as described above. A heuristic search was performed with 10 random sequence additions using likelihood as the optimality criterion.

Results

Sequence analyses

The final alignment of all genes in the analysis was 4224 base pairs (bp) in length. A total of 47 bp were excluded from the analysis as ambiguously aligned (see below). All analyses of the 73 ingroup plus outgroup taxa were conducted on the remaining 4177 characters.

Cyt*b* was 1143 bp in length in all but two taxa, *N. albicollis* and *C. enarratus*, which both showed a single codon deletion near the 3' end at positions 1135-1137. Further amplification and sequencing using different pairs of primers yielded similar results, suggesting the products were from authentic mtDNA rather than nuclear copies of mitochondrial DNA sequences. The fact that the deletions maintain the reading frame suggests the sequences were from functional coding sequences. Length variation at the 3' end of cyt*b* has been known to occur in other birds (Groth 1998; Cicero and Johnson 2001; Randi et al. 2001).

C-*myc* sequences ranged from 1230-1271 bp in length which totaled to 1316 bp in length when aligned. Of these, two poly-nucleotide tracts totaling 47 bp (29 in intron and 18 in UTR) were subsequently excluded from all analyses as too variable to be unambiguously aligned, resulting in 1269 bp aligned for analysis (range 1211-1241 bp unaligned). Of these, 323 bp (range 290-310) were from intron b, 575 bp (range 563-575) were from exon 3, and 371 bp (range 356-363) were from 3'UTR.

The remaining 1765 bp in the alignment were from GH. Raw sequences ranged from 781-1594 bp in length. 1659 bp (range 675-1488) were from intron 2, 32 bp were from exon 2, and 74 bp were from exon 3. Sequence length ranged from 781-923 bp for

all but 17 taxa, which had a sequence length of 1566-1594 bp due to the presence of a long insertion of ~769 bp in the intron.

Base compositions were homogeneous across all taxa and all genes when calculated based on parsimony informative characters (Table 4). However, when all characters were analyzed, base compositions were significantly heterogeneous for GH. This is likely due to the presence of the large insertion found in the intron of 17 of the Old World caprimulgids. When the insertion, which was rich in A's and G's, was removed from character analysis, base compositions were not significantly heterogeneous (Table 4). Cyt*b* was low in G's and T's, particularly in third base positions, and high in C's. C-*myc* was low in T's in the exon and low in C's and G's in the UTR. GH exons were low in G's.

Comparisons of mean pairwise distances (Figure 5) of taxa show that cyt*b* is evolving the fastest and c-*myc* the slowest, with GH intermediate between the other two genes. Within cyt*b*, third base positions are evolving at a much faster rate than first and second base positions. Exon 3 and the 3'UTR of c-*myc* are evolving at about the same rate, and both are evolving much slower than intron b. Exons of GH are evolving at a slightly slower rate than the intron.

The degree of substitution saturation in various gene elements was investigated by plotting the proportion of substitutions (total number of pairwise substitutions divided by total sequence length) against likelihood distance for c-myc, the most slowly evolving of the three genes (Figure 5). Both nuclear genes, c-*myc* and GH, were not saturated. Among cyt*b* sequences, there was evidence of saturation at all codon positions

Table 4: Characteristics of gene regions

Gene	BASE COMPOSITIONS [a]				χ^2 p-value	# parsimony informative characters	Average # of characters
	A	C	G	T			
Cyt*b*	0.33 (0.28)	0.46 (0.34)	0.05	0.16 (0.25)	1.00 (1.00)	506	1143
Cyt*b* 1+2	0.24 (0.23)	0.43 (0.28)	0.10	0.22 (0.31)	1.00 (1.00)	145	762
Cyt*b* 3	0.36 (0.39)	0.47 (0.45)	0.03	0.14 (0.13)	0.33 (0.53)	361	381
c-*myc*	0.26 (0.30)	0.25 (0.22)	0.29	0.20 (0.25)	1.00 (1.00)	200	1221
c-*myc* exon 3	0.24 (0.34)	0.31 (0.24)	0.28	0.16 (0.17)	1.00 (1.00)	63	563
c-*myc* intron b	0.26 (0.24)	0.24 (0.21)	0.29	0.21 (0.29)	1.00 (1.00)	98	300
c-*myc* 3'UTR	0.29 (0.31)	0.20 (0.19)	0.18	0.33 (0.32)	1.00 (1.00)	39	358
GH	0.21 (0.24)	0.27 (0.26)	0.25	0.26 (0.26)	1.00 (<	358	1007
GH exons 2 + 3	0.26 (0.34)	0.33 (0.28)	0.03	0.38 (0.20)	1.00 (1.00)	15	106
GH intron 2	0.21 (0.23)	0.27 (0.25)	0.26	0.26 (0.27)	1.00 (<	343	901
GH w/o insertion	0.20 (0.24)	0.28 (0.26)	0.25	0.27 (0.27)	1.00 (1.00)	294	830
GH intron 2 w/o	0.20 (0.23)	0.28 (0.26)	0.26	0.26 (0.28)	1.00 (1.00)	279	724
GH insertion only	0.32 (0.22)	0.19 (0.22)	0.32	0.17 (0.22)	1.00 (1.00)	64	758

[a] Base compositions shown are for parsimony informative characters only. Numbers in parentheses represent base compositions from all characters.

[b] P-values are for the χ^2 test of homogeneity of base compositions

Table 5: Average uncorrected pairwise distances

	cytb			c-myc				GH		
	All bp	1st + 2nd bp	3rd bp	All bp	Exon 3	intron b	3' UTR	All bp	Exons 2+ 3	Intron 2
All taxa	0.13	0.05	0.31	0.03	0.02	0.06	0.02	0.07	0.03	0.07
Outgroup only	0.18	0.08	0.38	0.06	0.04	0.12	0.05	0.12	0.04	0.13
Ingroup only	0.13	0.04	0.30	0.02	0.02	0.05	0.01	0.05	0.02	0.06
Ingroup to outgroup	0.17	0.07	0.38	0.06	0.04	0.12	0.04	0.13	0.05	0.14

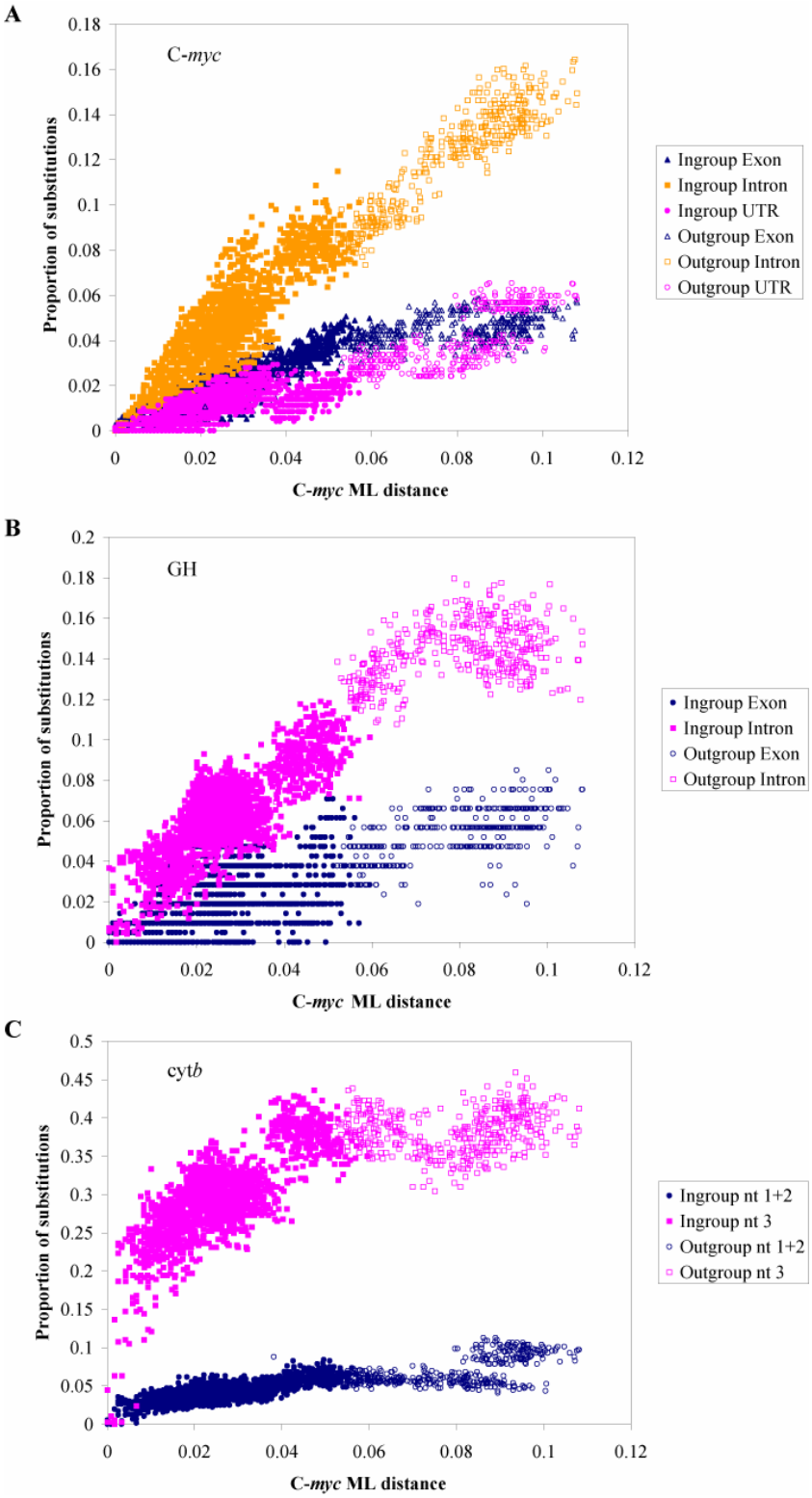


Figure 5: Saturation plots for sub-elements of each gene.

Proportion of substitutions (total number of pairwise substitutions divided by total sequence length) is plotted vs c-*myc* ML distance based on the optimized likelihood model (Table 3): (A) c-*myc*; (B) GH; and (C) cyt*b*.

(Figure 5C). In general, substitution types for all genes were biased towards transitions. For cyt*b*, the number of transitions appears to be decreasing as distances get larger. A closer examination of transitions and transversions versus likelihood distance indicates that at greater genetic distances, there are as many apparent transversions as there are transitions in third base positions (Figure 6B). At these distances, multiple hits have occurred at many sites and substitution saturation for cyt*b* may obscure phylogenetic signal.

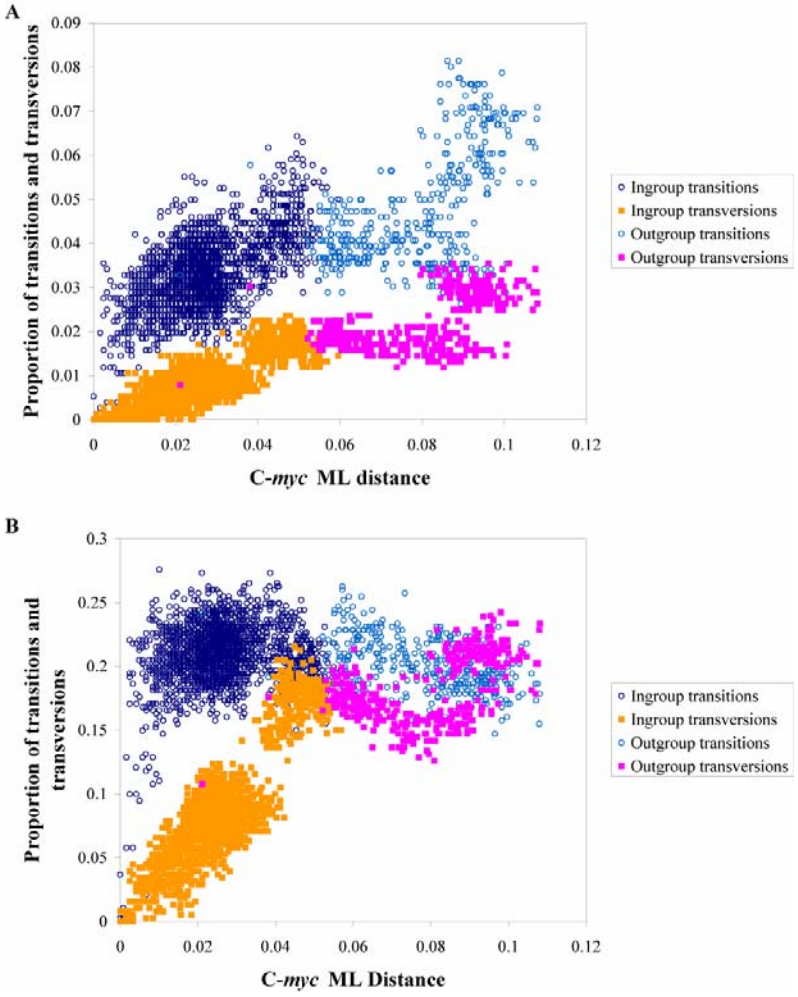


Figure 6: Saturation plot for cyt*b*:
(A) 1st and 2nd base position; (B) 3rd base position. Proportion of transitions and transversions are plotted against c-*myc* likelihood distance.

Phylogenetic Analyses

Incongruence Length Difference Tests

An ILD test of all data indicated significant conflict in phylogenetic signal among the genes (p = 0.02). Pairwise analyses indicated that GH was the source of the conflict (Table 6). To try to identify the source of conflict, several groups of taxa with different placement in the single gene trees were removed, and the ILD tests repeated, but the results remained significant. Due to the significant ILD test results, separate phylogenetic analyses were conducted on each gene, and on a combined cyt*b* and c-*myc* dataset. For comparison, all three datasets were also combined for analysis, despite the significant conflict of GH with the other gene partitions, because some studies suggest the ILD test may be too conservative (Bull et al. 1993; Cunningham 1997; Darlu and Lecointre 2002).

Table 6: P values for pairwise ILD test of gene congruence.

	Cytb	**c-myc**
c-myc	0.38	-
GH	0.01	0.01

Caprimulgid monophyly and basal taxa

Since all well supported nodes are found with each analysis method, only the ML tree is shown. Please refer to the Appendices for trees from MP and MB analyses.

Caprimulgidae is monophyletic with strong support in all analyses of both nuclear genes and all combined analyses (Figure 7-9, 11). In analyses of cyt*b* (Figure 10), three

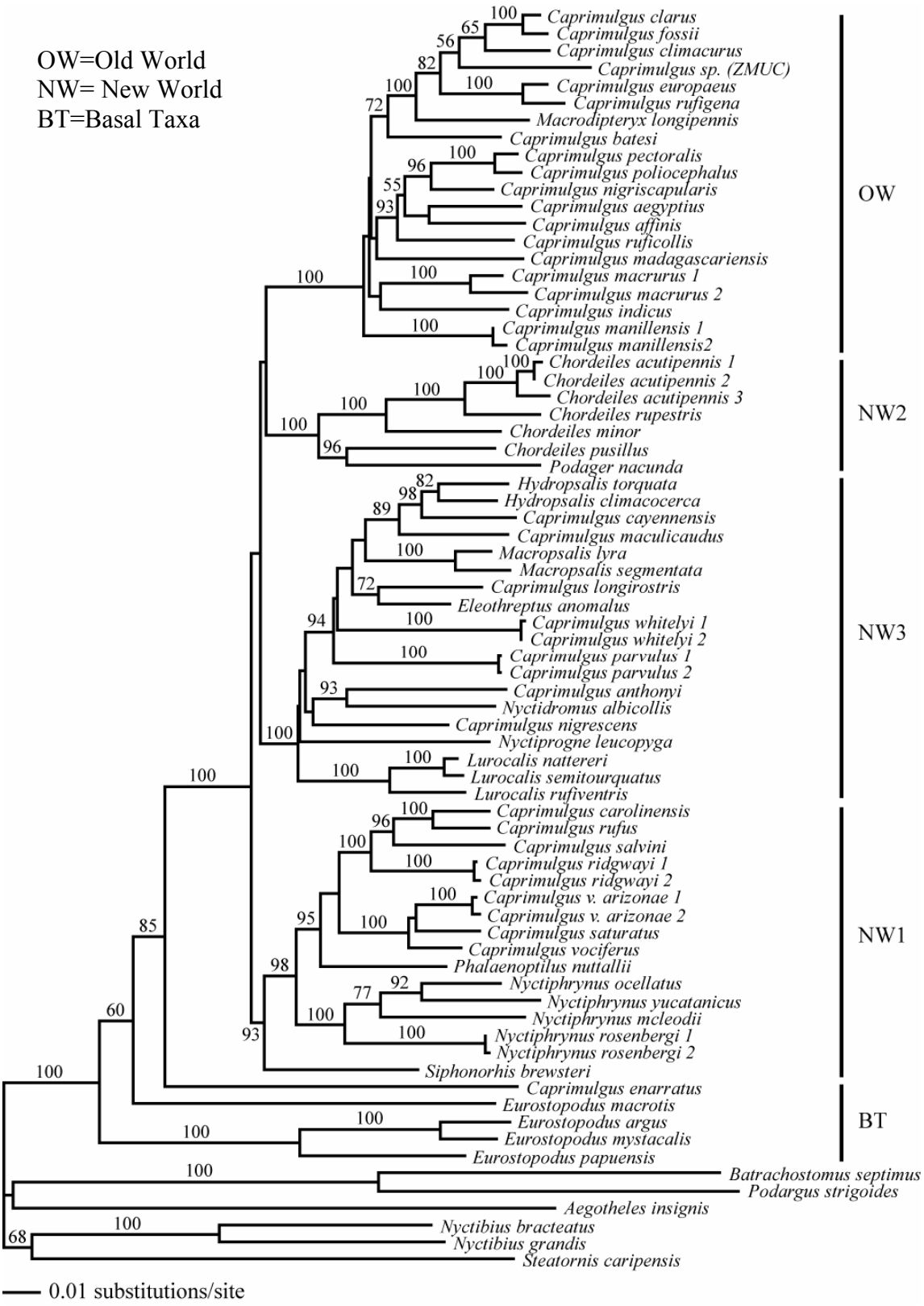


Figure 7: Maximum likelihood tree of all three genes combined (−ln L = 35807.86). Numbers indicate likelihood bootstrap (BP) >50%.

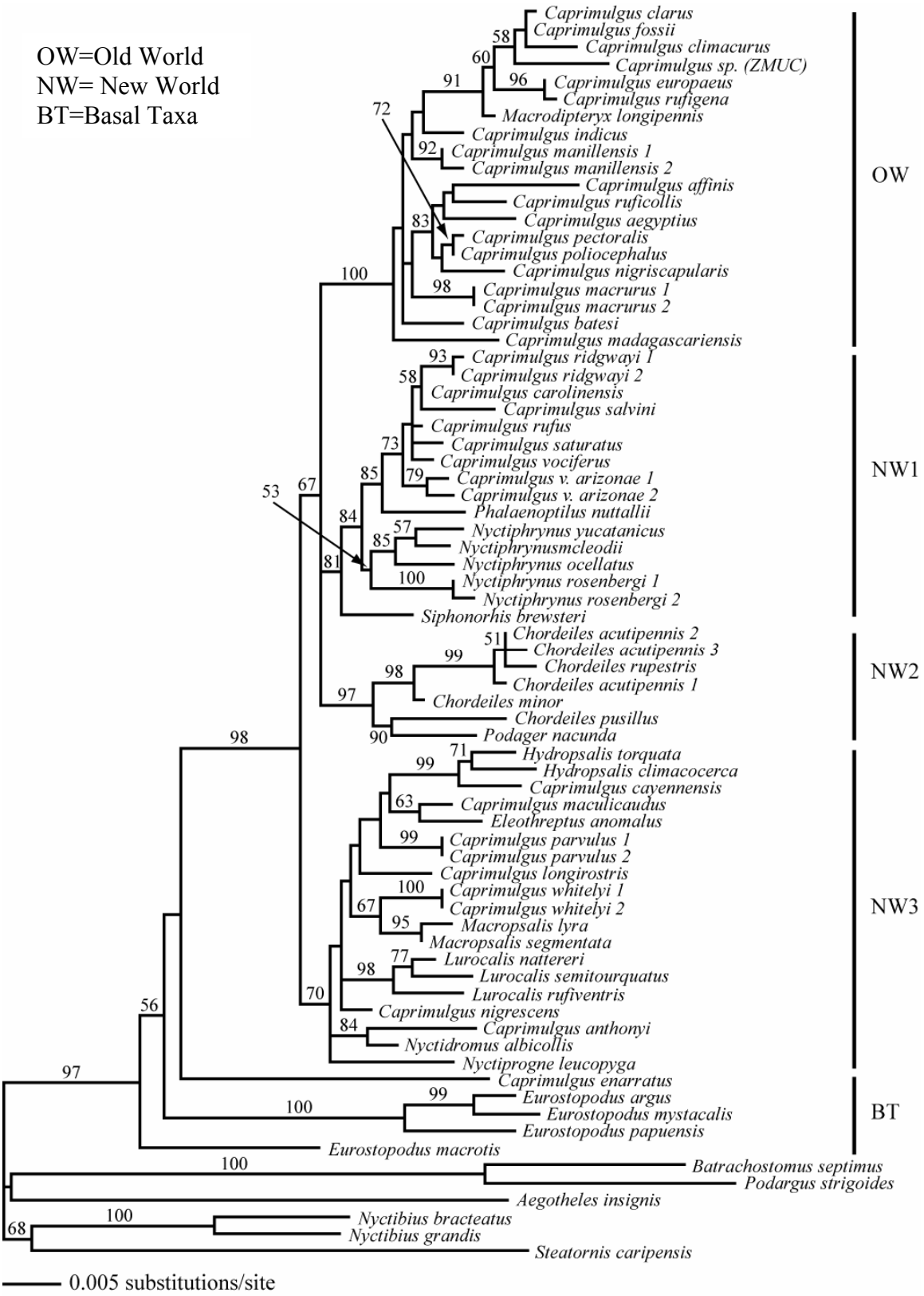


Figure 8: C-myc maximum likelihood tree (−ln L = 5933.29). Numbers indicate likelihood BP >50%

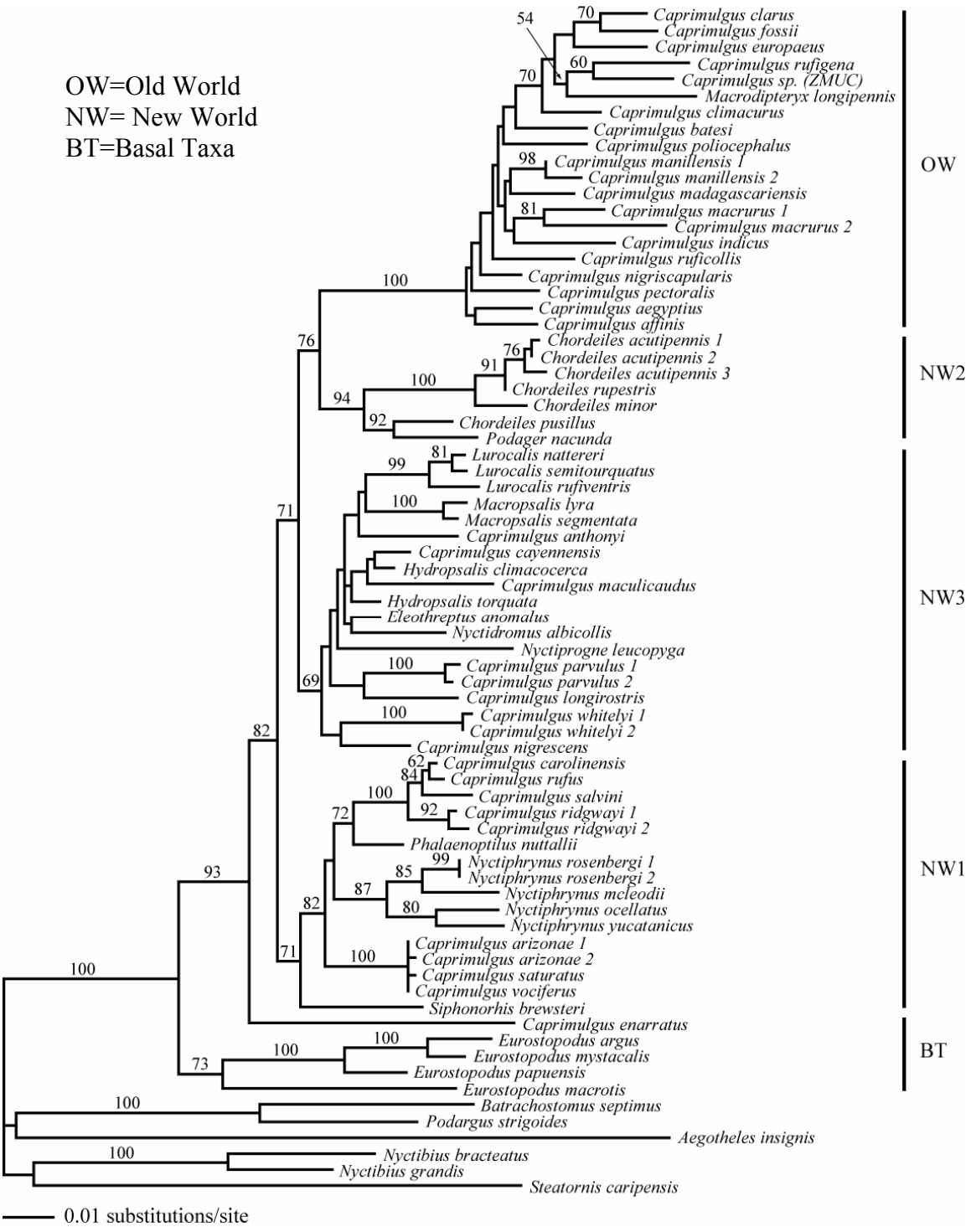


Figure 9: GH maximum likelihood tree (−ln L = 10101.34). Numbers indicate likelihood BP >50%.

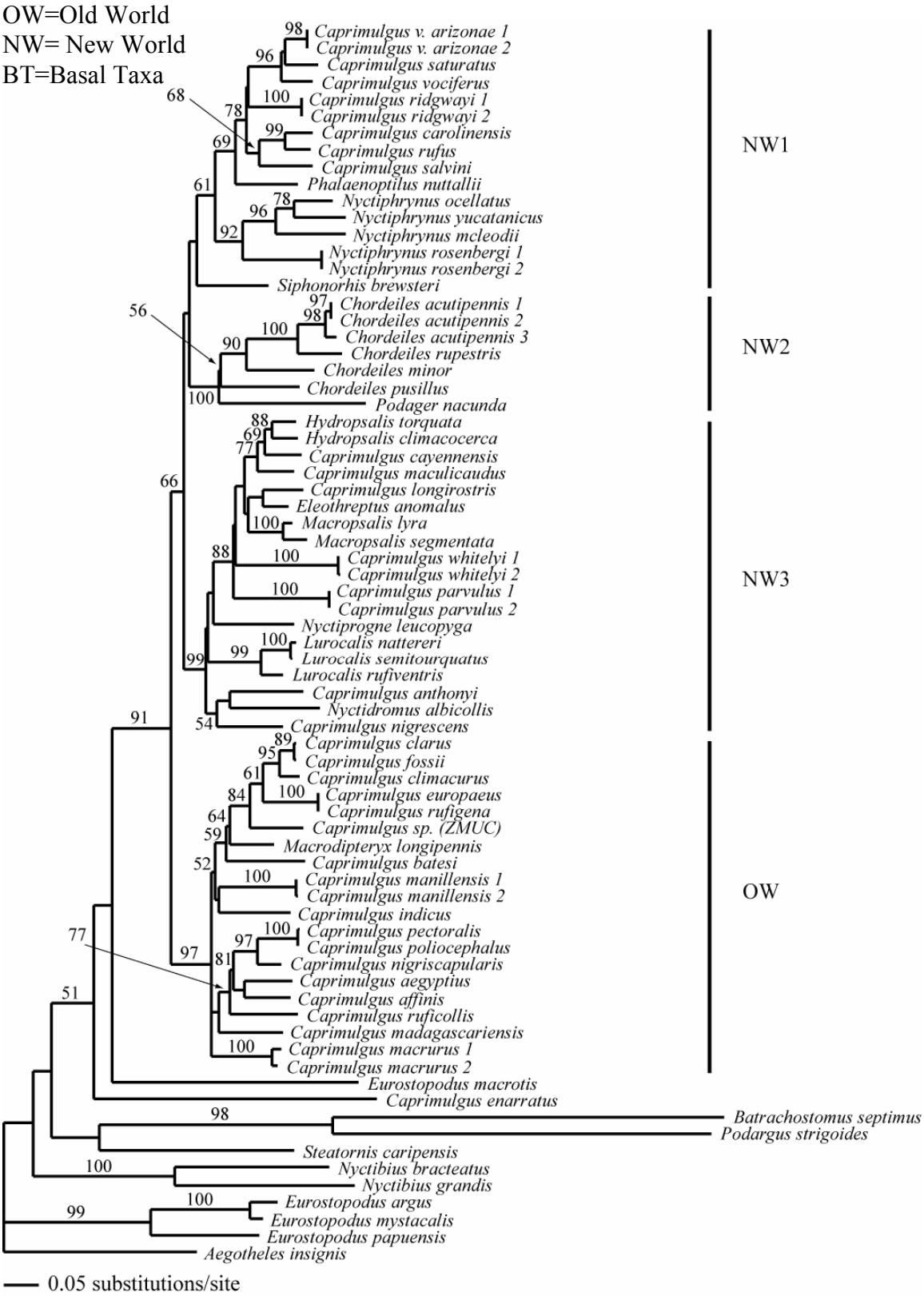


Figure 10: Cyt*b* maximum likelihood tree (-ln L = 18130.75).
Numbers indicate likelihood BP >50%

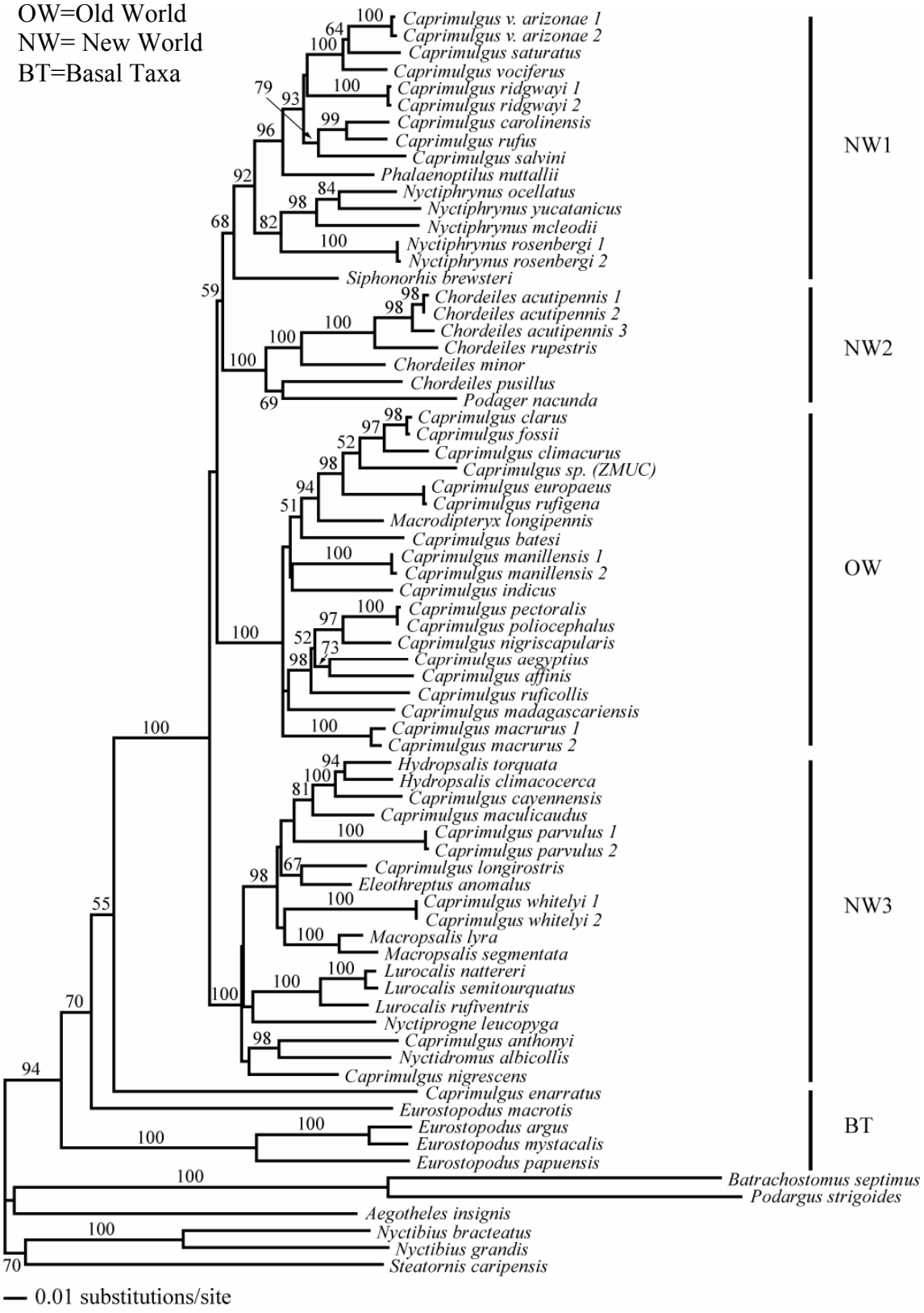


Figure 11: Cyt*b* + c-*myc* maximum likelihood tree. (−ln L = 24794.69). Numbers indicate likelihood BP >50%

Eurostopodus spp. fail to group together with the other Caprimulgidae. However, these are the most divergent ingroup taxa for all genes (Table 7), and substitution saturation in cyt*b* may be obscuring phylogenetic signal at that depth of the tree (Figure 6).

The four *Eurostopodus spp.* and *C. enarratus* are the earliest branching caprimulgids in all analyses (Figure 7-11). This group of five species are therefore referred to as "basal taxa" (BT). Genetic distances of these taxa to other Caprimulgidae are higher (Table 7), supporting the idea that these taxa are older than, and basal to, the rest of the Caprimulgidae.

There are two distinct groups within the genus *Eurostopodus*: one with *E. macrotis* and another with *E. papuensis*, *E. argus* and *E. mystacalis.* The average p-distance between the two groups is 0.10 whereas the average p-distance amongst the three *Eurostopodus* spp. (*papuensis, argus* and *mystacalis*) is 0.05. The order of branching of the two groups varies among genes and in combined analyses, without strong support for any of the possible topologies. The two groups cluster together in GH analyses, but with low support (Figure 9), and *Eurostopodus* is not monophyletic in cyt*b*, c-*myc* and combined analyses (Figure 7, 8, 10, 11). Therefore, monophyly of this genus remains unresolved.

C. enarratus is also an early branching taxon in all analyses (Figure 7-11). It is endemic to dense forests in NW and E Madagascar and is likely an old lineage. The position of *C. enarratus* on all trees refutes *Caprimulgus* monophyly.

Core Caprimulgids

All other caprimulgids form a clade with strong support in all analyses. ML

Table 7: Average uncorrected pairwise distances among groups of taxa

	cytb			c-myc				GH		
	All bp	1st+2nd bp	3rd bp	All bp	Exon 3	intron b	3' UTR	All bp	Exons 2+ 3	Intron 2
C. enarratus [a]	0.16	0.06	0.36	0.04	0.04	0.07	0.02	0.07	0.07	0.08
E. macrotis [a]	0.16	0.05	0.38	0.04	0.03	0.08	0.01	0.08	0.04	0.09
E. argus [a]	0.18	0.06	0.40	0.04	0.03	0.08	0.02	0.09	0.07	0.09
E. mystacalis [a]	0.17	0.07	0.38	0.04	0.04	0.08	0.02	0.09	0.08	0.09
E. papuensis [a]	0.16	0.06	0.37	0.04	0.03	0.09	0.02	0.08	0.07	0.08
Old World (OW) [b]	0.10	0.03	0.23	0.01	0.01	0.02	0.01	0.03	0.02	0.03
New World 1 (NW1)[b]	0.10	0.03	0.24	0.01	0.01	0.03	0.00	0.03	0.03	0.03
New World 2 (NW2)[b]	0.10	0.03	0.25	0.01	0.01	0.03	0.01	0.03	0.01	0.03
New World 3 (NW3) [b]	0.10	0.03	0.25	0.01	0.01	0.03	0.00	0.03	0.03	0.03
Basal taxa (BT) [b]	0.15	0.06	0.35	0.03	0.02	0.07	0.01	0.06	0.04	0.07
BT v outgroup (OG) [c]	0.18	0.07	0.39	0.06	0.04	0.13	0.04	0.12	0.09	0.14
OW to OG [c]	0.17	0.07	0.38	0.06	0.05	0.12	0.04	0.14	0.11	0.15
NW1 to OG [c]	0.17	0.07	0.38	0.06	0.04	0.12	0.04	0.13	0.10	0.14
NW2 to OG [c]	0.18	0.07	0.40	0.06	0.04	0.12	0.04	0.13	0.09	0.15
NW3 to OG [c]	0.18	0.07	0.38	0.06	0.04	0.12	0.04	0.12	0.09	0.13
BT to OW [c]	0.16	0.06	0.37	0.04	0.04	0.08	0.02	0.09	0.08	0.10
NW1 to OW [c]	0.13	0.04	0.30	0.02	0.02	0.04	0.01	0.06	0.07	0.06
NW2 to OW [c]	0.13	0.04	0.32	0.03	0.02	0.04	0.02	0.06	0.05	0.07
NW3 to OW [c]	0.13	0.04	0.30	0.03	0.02	0.04	0.02	0.06	0.05	0.06

[a] Average uncorrected p-distance for each of these taxa vs core caprimulgids

[b] Average uncorrected p-distance within each of the major groups of taxa found in analyses

[c] Average uncorrected p-distance between groups of taxa

Table 7 cont'd: Average uncorrected pairwise distances among groups of taxa

	cytb			c-myc				GH		
	All bp	1st+2nd bp	3rd bp	All bp	Exon 3	intron b	3' UTR	All bp	Exons 2+ 3	Intron 2
NW1 to BT [c]	0.17	0.06	0.38	0.04	0.03	0.08	0.01	0.08	0.06	0.08
NW2 to BT [c]	0.17	0.06	0.40	0.04	0.03	0.08	0.01	0.08	0.06	0.09
NW3 to BT [c]	0.17	0.06	0.38	0.04	0.03	0.08	0.02	0.08	0.06	0.08
NW1 to NW2 [c]	0.13	0.04	0.31	0.02	0.02	0.05	0.01	0.06	0.04	0.06
NW1 to NW3 [c]	0.12	0.04	0.29	0.02	0.01	0.05	0.01	0.05	0.04	0.05
NW2 to NW3 [c]	0.13	0.04	0.31	0.03	0.02	0.06	0.02	0.05	0.03	0.06

[a] Average uncorrected p-distance for each of these taxa vs core caprimulgids

[b] Average uncorrected p-distance within each of the major groups of taxa found in analyses

[c] Average uncorrected p-distance between groups of taxa

bootstrap support for this core caprimulgid clade ranged from 82-100% (Figure 7-11). Within this clade, there are four main groups found consistently in all single gene analyses, but with varying degrees of support (Figure 8, 9, 11). These groups include one Old World (OW) and three New World (NW) clades. When the genes are combined, support for each of these four main groups is strong (Figure 7, 11). However, the relationships among these four groups remain unresolved, as the exact topology varies among the genes and in combined analyses.

Four core clades

Old World

This clade includes all African, Asian, and European taxa sampled, aside from the basally branching *Eurostopodus spp*. and *C. enarratus*. Support for it is very strong ranging from 97-100% ML bootstrap in single gene and combined analyses (Figure 7-11). A clade comprising *C. clarus, C. fossii*, *C. climacurus*, *C. europaeus, C. rufigena,* the unidentified *Caprimulgus sp.* from ZMUC, and *M. longipennis* is present in all analyses (ML bootstrap 64-94%) with support becoming very strong when all three genes are combined (100% ML bootstrap). *C. clarus* is sister to *C. fossii* in all analyses except in c-*myc* (Figure 8) where it forms a polytomy with *C. climacurus*. All three taxa form a clade in all analyses except GH (Figure 9). At one time, these three taxa were classified under a separate genus, *Scotornis* (Peters 1940). *C. europaeus* is sister to *C. rufigena* with strong support in all analyses. The unidentified *Caprimulgus* sp. from ZMUC is not one that has been sequenced in this study. Based on collection locality, some possible species are: *C. tristigma, C. natelensis, C. ruwenzorii,* or *C. fraenatus*.

New World 1

NW 1 is comprised of *Nyctiphrynus*, *Phalaenoptilus*, *Siphonorhis*, and a group of *Caprimulgus spp.* This group is primarily made up of poorwills, whip-poor-wills, and chuck-wills-widow. All *Caprimulgus* spp., with the exception of *C. rufus*, are found in North and Central America. *Siphonorhis*, a West Indian island endemic, is basal to all taxa within this group in all analyses except in cyt*b* and c-*myc* parsimony analyses (See Appendix D, E).

C. v. vociferus, C. v. arizonae, and *C. saturatus* form a group in all analyses except those based on c-*myc*, but their interrelationships remain unresolved. This suggests either that *C. v. arizonae* should be elevated to full species level rather than as a subspecies of *C. vociferus*, or that *C. saturatus* should be treated as a subspecies of *C. vociferus*.

The group *C. salvini* plus *C. carolinensis* and *C. rufus* is found with varying degrees of support in all analyses except those based solely on c-*myc* or GH. This relationship is stronger in combined analyses. This group, along with *C. ridgwayi*, forms a strong clade of species whose breeding distributions are non-overlapping. An unsampled species, *C. badius*, which breeds from Yucatan to Nicaragua, may complete this group.

New World 2

NW2 is comprised of two nighthawk genera, *Chordeiles* and *Podager*. This clade is formed with high support in all single gene and combined analyses. All analyses (Figure 7-10) place *P. nacunda* as sister to *C. pusillus* except for those based on cyt*b* (Figure 10) where instead, *P. nacunda* branches basally to all *Chordeiles* species. *C.*

acutipennis is sister to *C. rupestris* in all analyses, with the exception of c-*myc* where these two species form a polytomy with *C. minor*. The separation of this clade from *Lurocalis* and *Nyctiprogne* makes Chordeilinae non-monophyletic.

New World 3

NW3 is primarily found in South America, and comprises *Hydropsalis, Macropsalis, Eleothreptus, Nyctidromus, Nyctiprogne, Lurocalis* and *Caprimulgus*. Both nightjars (*Hydropsalis, Macropsalis, Eleothreptus, Nyctidromus* and *Caprimulgus*) and nighthawks (*Lurocalis* and *Nyctiprogne*) are found within this group. *Hydropsalis spp.* are sister to *C. cayennensis* in all analyses except GH, where the relationships are unresolved.

Constrained search results

As a test of monophyly of current genera and subfamilies, constrained tree searches were run to compare likelihoods of constrained tree topologies to the likelihoods of optimal unconstrained tree topologies. Monophyly of various groups (*Eurostopodus, Caprimulgus*, Chordeilinae, Caprimulginae) was enforced in separate constrained tree searches (Table 8). Monophyly of both the traditional and Holyoak's subfamilies were tested. The results indicate a large decrease in likelihood when the genus *Caprimulgus* was constrained to be monophyletic, and when each of the subfamilies, either with Holyoak's definition or with the traditional classification, were constrained to be monophyletic. There was, however, a very small decrease in likelihood when *Eurostopodus* was constrained to be monophyletic (Table 8). These results indicate that monophyly of *Caprimulgus*, Chordeilinae and Caprimulginae are all strongly rejected by the data, while the evidence against *Eurostopodus* monophyly is less strong.

Table 8: Comparison of likelihoods of alternative tree topologies

Hypothesis	Cyt b	c-myc	GH	Combined
Holyoak Chordeilinae monophyletic[a]	120.36	94.28	92.52	288.65
Chordeilinae monophyletic[b]	36.99	26.72	21.09	76.14
Holyoak Caprimulginae monophyletic[c]	83.19	73.50	67.23	214.78
Caprimulginae monophyletic[d]	106.37	97.09	105.54	304.09
Eurostopodus monophyletic[e]	8.18	0.70	-	2.86
Caprimulgus monophyletic[f]	258.74	200.38	221.03	673.37

Constrained ML heuristic tree searches were conducted in PAUP* using models of evolution previously estimated for unconstrained tree searches (Table 3) with parameters optimized using Modeltest 3.7. Each constrained tree was compared to the respective optimal unconstrained ML tree for (a) cyt*b* (-ln L = 18130.75), (b) c-*myc* (-ln L = 5933.29), (c) GH (-ln L = 10101.34), and (d) total combined datasets (-ln L 35807.86). Δln L values shown are the decreases in likelihood of the constrained versus the unconstrained trees.

[a] ((*Chordeiles* [6 OTUs], *Eurostopodus* [4 OTUs], *Lurocalis* [3 OTUs] *Nyctiprogne*))

[b] ((*Chordeiles* [6 OTUs], *Lurocalis* [3 OTUs] *Nyctiprogne* , *Podager*))

[c] ((*Caprimulgus spp.* [37 OTUs], *Eleothreptus, Hydrosalis* [2 OTUs], *Macrodipteryx, Macropsalis* [2 OTUs], *Nyctidromus, Nyctiphrynus* [5 OTUs] *P. nuttallii, P. nacunda, S. brewsteri*))

[d] ((*Caprimulgus spp.* (37 OTUs), *E. anomalus, E. argus, E. macrotis, E. mystacalis, E. papuensis, H. climacocerca, H. torquata, M. longipennis, M. lyra, M. segmentata, N. albicollis, N. mcleodii, N. ocellatus, N. rosenbergi* (2 OTUs), *N. yucatanicus, P. nuttallii, S. brewsteri*))

[e] ((*E. argus, E. macrotis, E. mystacalis, E. papuensis*))

[f] ((37 *Caprimulgus spp.))*

Indel support

There was a total of 122 indel characters using simple indel coding implemented in SeqState (Müller 2006). Of these, 74 characters were autapomorphic and four characters were indels located in poly-T regions that are prone to length variation and are therefore highly homplasious. Fourteen indel characters were homoplasious requiring two to three changes on the combined ML tree. The remaining thirty informative indel characters, requiring only a single change on the tree, were mapped onto the combined ML tree (Figure 12). Most of these indels mapped to nodes that are strongly supported in analyses of substitutional variation. However, several provide valuable additional support for weak or unresolved nodes. For example, a large insertion (769 bases) is present in most OW taxa with the exception of *C. manillensis* and *C. madagascariensis*. There is little resolution at the base of the OW clade in any of the trees based on substitutional variation, therefore the 769 bp insertion provides good evidence for uniting all taxa in which it is present, and placing *C. manillensis* and *C. madagascariensis* at the base of the clade. In our trees, the two taxa do not form a clade but are separated by short internodes with no support.

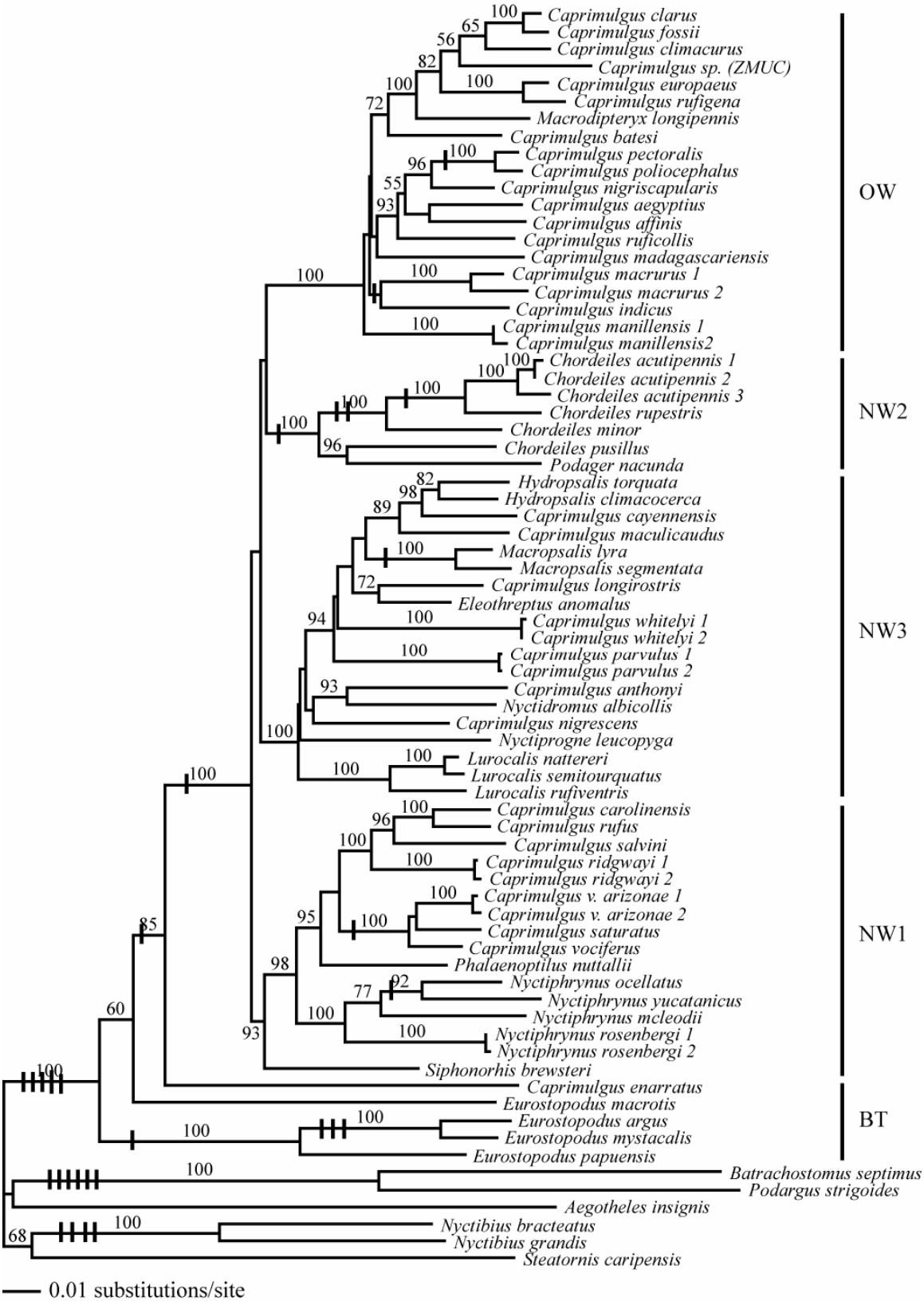


Figure 12: Combined ML tree with indel characters indicated by black bars. Numbers indicate likelihood bootstrap >50%

Discussion

Hypotheses and general conclusions

The genetic data presented here confirm some traditional groupings of caprimulgids and provide a host of new insights. All Caprimulgidae are monophyletic with respect to outgroup taxa. Within the family, the two subfamilies, Caprimulginae and Chordeilinae, are not monophyletic. Two of the four traditional chordeiline genera, *Chordeiles* and *Podager*, form the NW2 clade while the other two genera, *Lurocalis* and *Nyctiprogne*, are found within the NW3 clade with several traditionally caprimulgine genera. The support for both clades is consistent among the three genes and strong in combined analyses. In addition, *Eurostopodus* is an early radiation among all other Caprimulgidae, making the traditional Caprimulginae non-monophyletic.

The alternative groupings of subfamilies, according to Holyoak (2001), are also not monophyletic. *Podager*, which Holyoak considered to be caprimulgine, groups together with *Chordeiles* in the NW2 clade. This suggests that rictal bristles, which Holyoak used to define the subfamilies, are not good diagnostic characters. The genus *Veles*, treated as chordeiline by Holyoak (2001), was not available for study. This genus has often been lumped with *Caprimulgus,* and presumably would group within the OW clade, but that must remain speculation until genetic data are available. Maximum likelihood tree searches, where each of the subfamilies (both traditional and Holyoak's) was constrained to be monophyletic, resulted in very large decreases in likelihood, rejecting monophyly of each subfamily. Non-monophyly of the subfamilies suggest that aerial foraging, and the associated morphological adaptations, arose more than once (see *Character Evolution* below).

According to the genetic data, *Eurostopodus* spp. forms the base of all Caprimulgidae. However, monophyly of *Eurostopodus* was inconclusive. The ML tree with combined data has *Eurostopodus* paraphyletic, and the two groups have large genetic distances between them. Indel data support *E. argus, E. papuensis,* and *E. mystacalis* as being more closely related to each other than to *macrotis*, which differs from the other three species in possessing ear tufts. These results clearly indicate that *macrotis* is divergent enough from the other *Eurostopodus spp.* to warrant being placed into a separate genus *Lyncornis*. However, constraint tree searches (Table 8) resulted in only a small decrease in likelihood to make the genus monophyletic. These results, while consistent with Sibley and Ahlquist's (1990) DNA hybridization study in showing large genetic distances, cast doubt on their recommendation to make it a separate family, Eurostopodidae. If that course were taken, consistent treatment would require separation of *macrotis* and its probable sister species, *temminckii*, in yet another small family Lyncornidae. That would serve little purpose in conveying the presently inferable relationships among nightbirds. Although Barrowclough et al (2006) concurred with Sibley and Ahlquist on the separation of Eurostopodidae, limited taxon sampling prevented them from detecting the deep split within the current genus *Eurostopodus*. Only one species, *Eurostopodus* (=*Lyncornis*) *macrotis*, was included in their study.

In all analyses, *Caprimulgus* is polyphyletic, with species found in each of the major clades with strong support. ML tree searches, where this genus was constrained to be monophyletic, resulted in very large decreases in likelihood. *Caprimulgus* has traditionally been a catchall for all Caprimulgidae lacking in striking characters, and the need to revise the genus has been remarked upon (Sibley and Ahlquist 1990; Cleere

1998; Cleere 1999; Holyoak 2001). Polyphyly indicates caprimulgids have maintained a successful body plan, while alternative forms arose from within the traditional generic limits. A number of genera have been named in the past, some of which may still be valid.

Biogeography

In this study, the five early branching taxa, *Eurostopodus spp.* and *C. enarratus*, are found in Australasia and Madagascar suggesting this region around the Indian Ocean as a possible origin of Caprimulgidae. *C. enarratus*, endemic to dense forests in NW and E Madagascar, is an early branching taxon found at the base of all Caprimulgidae save *Eurostopodus*. One other species endemic to Madagascar, *C. madagascariensis*, is placed within the OW clade and has a more widespread distribution across the island, suggesting a second, more recent colonization.

The data give strong support for biogeographical groupings among the remaining core caprimulgids, with one clade comprising the majority of the Old World taxa and the other three clades being New World. While the interrelationships among these groups remain unresolved, all analyses, except those based solely on cyt*b*, indicate a radiation in the New World, from which a second Old World clade was derived. While more data would be needed to fully support this conclusion, it is consistent with the study of Barrowclough et al (2006) based on RAG-1. Those authors suggested a small genus, *Eurostopodus*, from Australasia, was sister to the rest of the family. Those authors also suggested a second Old World clade was derived from a larger New World clade.

Character evolution

Based on the results of this study, the characters used to define the traditional subfamilies are homoplasious (Figure 13). Many of these characters appear to be tracking the trophic niches of the taxa more closely than phylogeny. Rictal bristles, which Holyoak used to rearrange the traditional subfamilies, must have been gained and lost multiple times. They are likely to be the result of trophic adaptation to aerial foraging by sallying from a perch that occurred in the common ancestor of core caprimulgids and *C. enarratus*. Those lineages that forage by hawking insects on the wing (the nighthawk niche) have subsequently lost rictal bristles. These lineages would include *Nyctiprogne*, *Lurocalis* and *Chordeiles*. *Nyctiprogne* and *Lurocalis* are not strongly separated by the available genetic data. Their morphological similarities suggest that they may be sister taxa rather than basally paraphyletic in NW3 as shown in Figure 13. If so, only one transition to a nighthawk niche would be required in NW3. The Nacunda Nighthawk (*Podager*) is embedded within *Chordeiles* (NW2) yet has rictal bristles. If gain or loss of bristles is equally probable, it is equally parsimonious to suppose that *Podager* regained them after loss in the ancestor of NW2, or that *Podager* retained them as the primitive state while they were lost independently by *C. pusillus* and the ancestor of the other three *Chordeiles* species. However, if loss is more probable than gain, then the second explanation is more parsimonious.

Many of the genera containing only one or two species are named for striking morphological features such as modified wing or tail feathers (Figure 13). These traits are likely to be sexually selected and represent recent autapomorphies rather than deep phylogentic divergences. *Caprimulgus* polyphyly indicates that these characters have

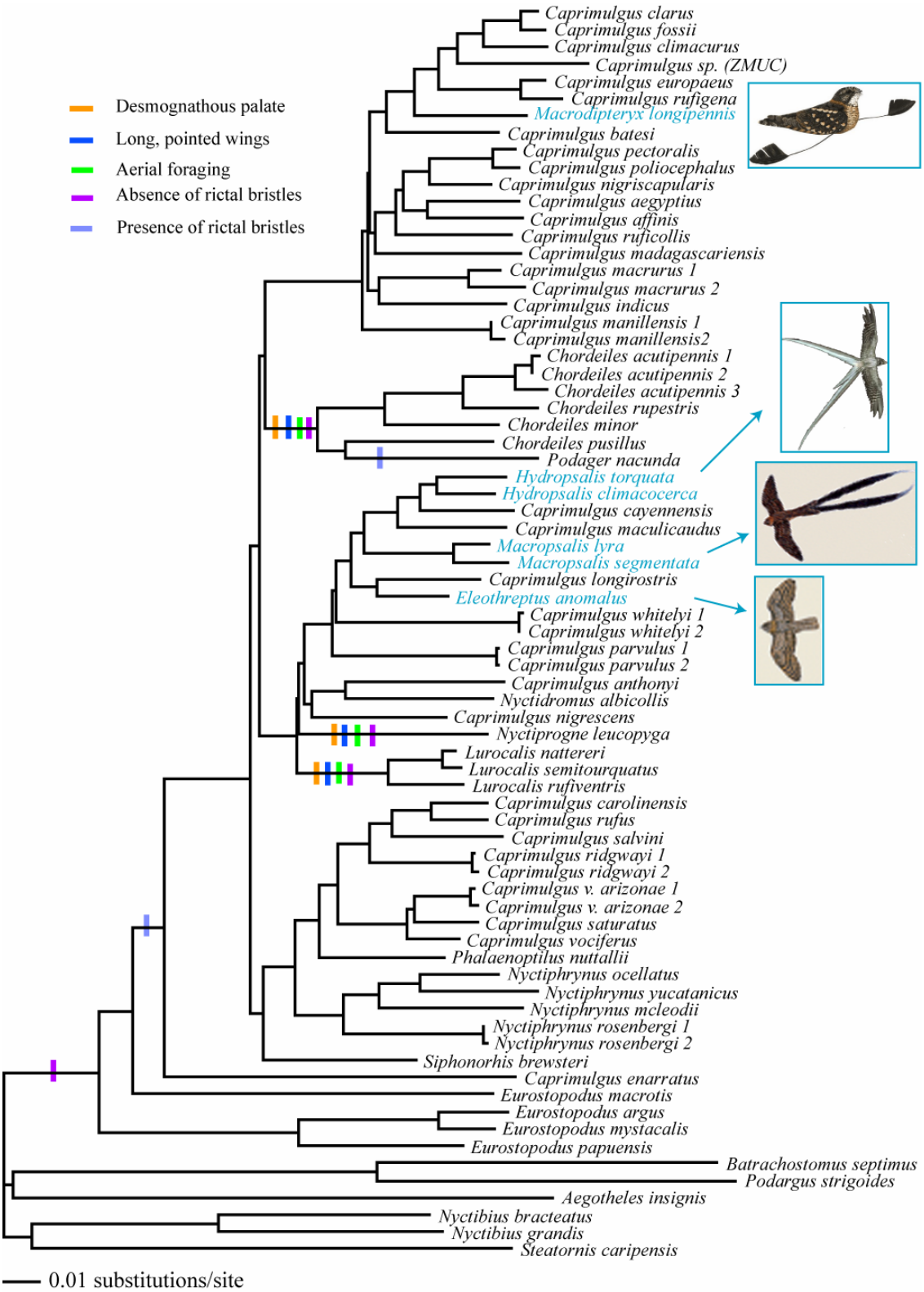


Figure 13: Combined ML tree with traditional morphological and behavioral characters mapped on tree.
Bars represent characters used to define the subfamilies. Pictures on right represent morphological characters used to define genera

evolved multiple times from a basic body plan. The elongated tail feathers of *H. torquata* and *Macropsalis* spp. provide a good example. Although all three fall in the same clade, they are separated by several robust nodes including taxa of normal tail length. Thus elongated tail feathers must have been gained or lost on more than one occasion.

Taxonomic conclusions

The results from this study were not consistent with the division of the family into two subfamilies, Chordeilinae and Caprimulginae. It would also be difficult to divide the family into two subfamilies, Eurostopodinae and Caprimulginae, as suggested by Barrowclough et al (2006), since monophyly of *Eurostopodus* is still uncertain. However, many monotypic genera can be reclassified and the species rich genus *Caprimulgus* can be subdivided.

Although *E. temminckii* was not sampled, it is likely sister to *E. macrotis* since the two species share morphological similarities such as the presence of "ear tufts". Both *E. temminckii* and *E. macrotis* were previously classified as *Lyncornis*, and this genus should be resurrected for the two species.

C. enarratus should be assigned to a new monotypic genus, while genus names within the core caprimulgids should be divided by the four major clades. *C. enarratus* is as divergent from the core caprimulgids as each of the four major clades are from each other. The OW clade should be assigned to the genus *Caprimulgus,* which would get rid of one genus, *Macrodipteryx* and possibly another, *Veles*. NW2 should be assigned to the genus *Chordeiles,* which appears to have priority over the monotypic genus *Podager* (Peters 1940). NW3 which has seven different genera would need to be assigned a genus name, possibly *Hydropsalis*, since it appears to have priority over other genera in the

clade (Peters 1940). Within NW1, *Siphonorhis*, which appears to be basal to the rest of the clade, could remain, as could *Nyctiphrynus*, which appears sister to the rest of the taxa within this clade. All other taxa would need to be assigned the genus *Antrostomus*, which was used previously for several taxa within this clade.

This reclassification would decrease the total number of genera from fifteen to nine. In addition, these generic designations would better reflect evolutionary history. The question remains of what to do with the remaining unsampled taxa. Based on the current information, guesses could be made as to which of the major clades any remaining taxa would belong. However, any of these unsampled taxa could turn out to form another major group, complicating the picture presented here. Until tissue samples of the remaining taxa become available, these results present a major advance in caprimulgid classification.

Appendices

Appendix A: Synopsis of Caprimulgiform systematics

Sclater (1866a; 1866b) subdivided the Caprimulgidae into Steatornithinae, Podarginae (consisting of *Podargus, Batrachostomus, Nyctibius,* and *Aegotheles*), and Caprimulginae. This subdivision was followed by Beddard (1886) who recognized four subfamilies based on the syrinx and other morphological characters. *Steatornis* was placed into its own subfamily, *Podargus* and *Batrachostomus* into a second subfamily, *Aegotheles* into a third subfamily and *Caprimulgus*, *Chordeiles,* and *Nyctidromus* into a fourth subfamily. Wetmore (1918) recognized Nyctibiidae as being a distinct group intermediate between Podargidae and Caprimulgidae based on morphological traits. Peters (1940), in his *Checklist of the Birds of the World*, following Wetmore, recognized five families, Aegothelidae, Podargidae, Steatornithidae, Nyctibiidae, and Caprimulgidae. He further subdivided Caprimulgidae into two subfamilies: Caprimulginae and Chordeilinae. This arrangement continues to be used today.

While most ornithologists recognize these five families as being each others' closest relatives, disagreements continue to exist at the ordinal level. Although they have been linked to other groups of birds in the past, Caprimulgiformes are most commonly treated as relatives of either owls (Strigiformes) or swifts and hummingbirds (Apodiformes), or both (Cleere 1998; Cleere 1999; Holyoak 2001) with some families more closely allied to Apodiformes and others to Strigiformes. DNA-DNA hybridization studies (Sibley and Ahlquist 1990; Bleiweiss et al. 1994) suggest that owls, and not swifts, are the closest living relatives of Caprimulgiformes.

More recently, monophyly of the order has been questioned. A study of osteological characters (Mayr 2002) found evidence of paraphyly in Caprimulgiformes.

Their results indicated Aegothelidae formed a monophyletic clade with Apodiformes and were sister to a monophyletic clade comprising of Caprimulgidae and Nyctibiidae. All four of these groups formed a monophyletic group with respect to the other Caprimulgiformes. Further molecular studies using cytochrome *b* and *c*-myc (Braun and Huddleston unpublished data), while confirming monophyly of each of the five families, failed to confirm monophyly of the order. Aegothelidae was found to be sister to Apodiformes.

Appendix B: Laboratory Methods

DNA Extraction

Tissues were either minced or ground under liquid nitrogen and digested at 60°C overnight in a shaking incubator with 50 µL of Proteinase K (20mg/mL), 75 µL of 20% Sodium Dodecyl Sulfate (SDS) and 1 ml of DNA Extraction Buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM disodium EDTA). The solution was then extracted twice with 1 ml of PCI (phenol:chloroform:isoamyl alcohol, 25:24:1 v/v) using Phase-Lock gel (Eppendorf) to maximize separation of the organic and aqueous phases. The resulting solution was then extracted twice with 1 ml of CI (chloroform:isoamyl alcohol, 24:1 v/v) to remove trace phenol. The DNA was precipitated with 1/10 the volume of 3 M NaOC (sodium acetate) and 2.5 volumes of ice-cold 95% ethanol (EtOH) and placed at −20°C overnight. The solution was frozen at −80°C for one hour, then the DNA was spun down in a 4°C centrifuge (Eppendorf Centrifuge 5415D) at maximum speed. The resulting pellet was rinsed twice with 1 mL of 70% ethanol. The DNA samples were dried to remove any traces of ethanol and re- suspended with 300 µL of TLE (10 mM Tris, 0.1 mM EDTA). Genomic DNA was visualized on a 1.5% agarose gel to check for quality and RNA. RNase was added if necessary. Concentrations were checked using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

PEG Precipitation

Excess nucleotides and primers were removed from PCR products by PEG precipitation. An equal volume of a solution of 20% PEG 8000, 2.5 M NaCl was added to the PCR product and incubated for 15 min at 37°C. The solution was spun for 15 min

in a cold centrifuge (Eppendorf Centrifuge 5415D) at maximum speed and the resulting supernatant removed. The pellet was then washed twice with 150 µL of 80% cold EtOH and centrifuged for five min between each wash. The pellet was dried to remove EtOH and re-suspended with 25-50 µL of dH_2O.

Sequencing

Cycle sequencing reactions were carried out in 10µL reactions with 0.5 µL Big Dye v.3.1 chemistry (Applied Biosystems) and 1.75 µL 5x Sequencing Buffer (Applied Biosystems). Sequencing conditions were as follows: 96°C for 1 min (90 seconds for GH); 45 cycles (40 cycles for GH) of 96°C for 10 sec, 50°C for 5 sec, and 60°C (55°C for GH) for 4 min.

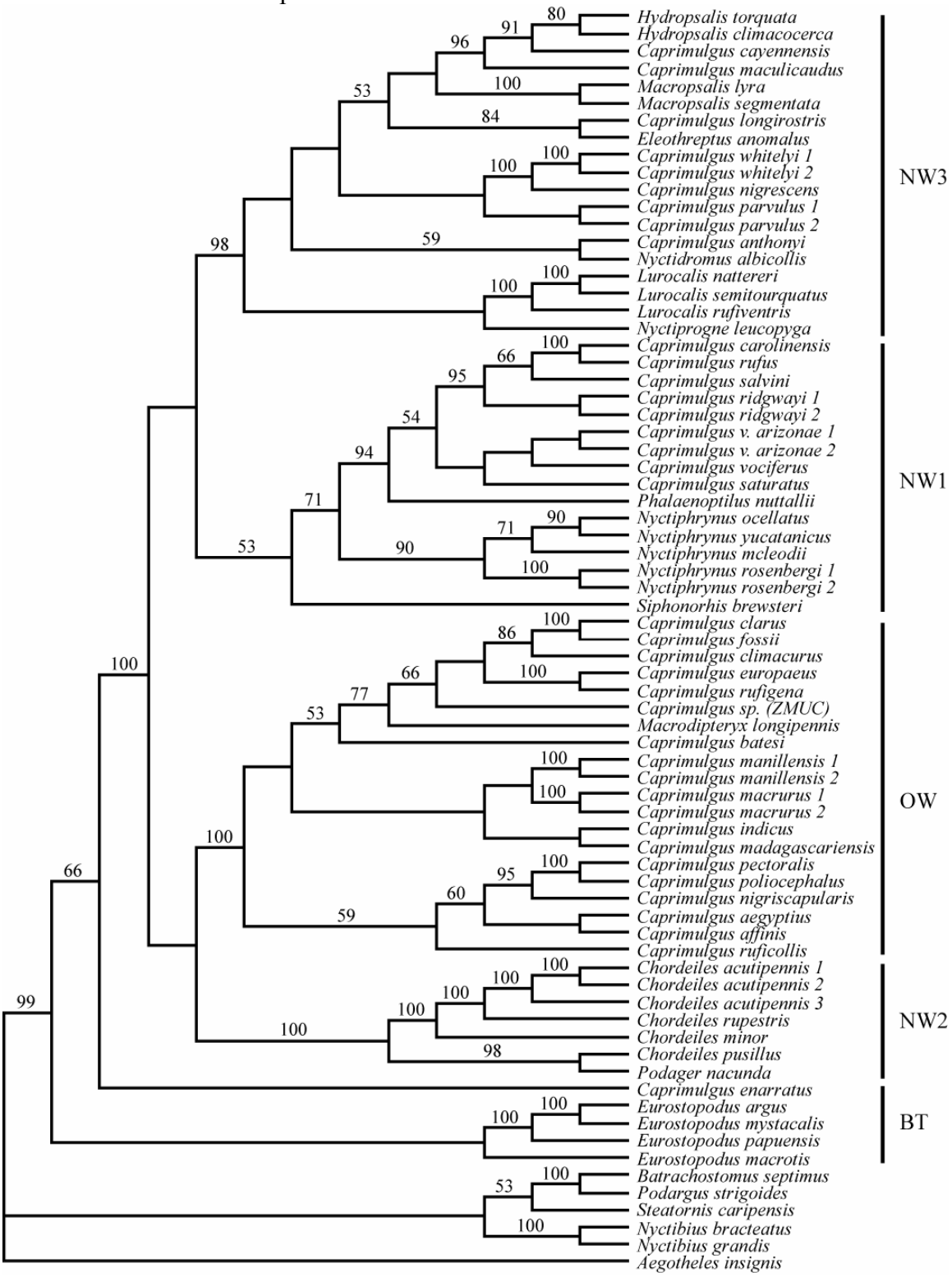
Cloning

Cloning reactions followed manufacturer's protocol but were reduced to ¼ volumes of those specified by the manufacturer (0.25 µL Vector, 0.25 µL Salt Solution, 1 µL PCR product). Only cells with insertions were ampicillin resistant and could grow on the agar plate, eliminating the need for blue-white screening. Eight to ten colonies were selected from each plate and double checked for insertion using PCR and standard M13 primers. Test reactions were performed in 10 µL volumes. Final concentrations were: 1x PCR Buffer (GeneChoice), 1.5 mM $MgCl_2$ (included with Buffer), 0.15 mM each dNTP, 0.5 µM each M13 forward and reverse primers, to which was added 1 unit *Taq* polymerase (GeneChoice), and cloned cells. The PCR test for insert cycling conditions were as follows: Initial denaturation at 95°C for 3 min; followed by 30 cycles of 95°C for 15 sec, 50°C for 15 sec and 72°C for 1 min; and a final extension at 72°C for 10 min.

Products were visualized on a 1.5% agarose gel. Two positive colonies were selected from each plate and grown overnight in LB liquid culture.

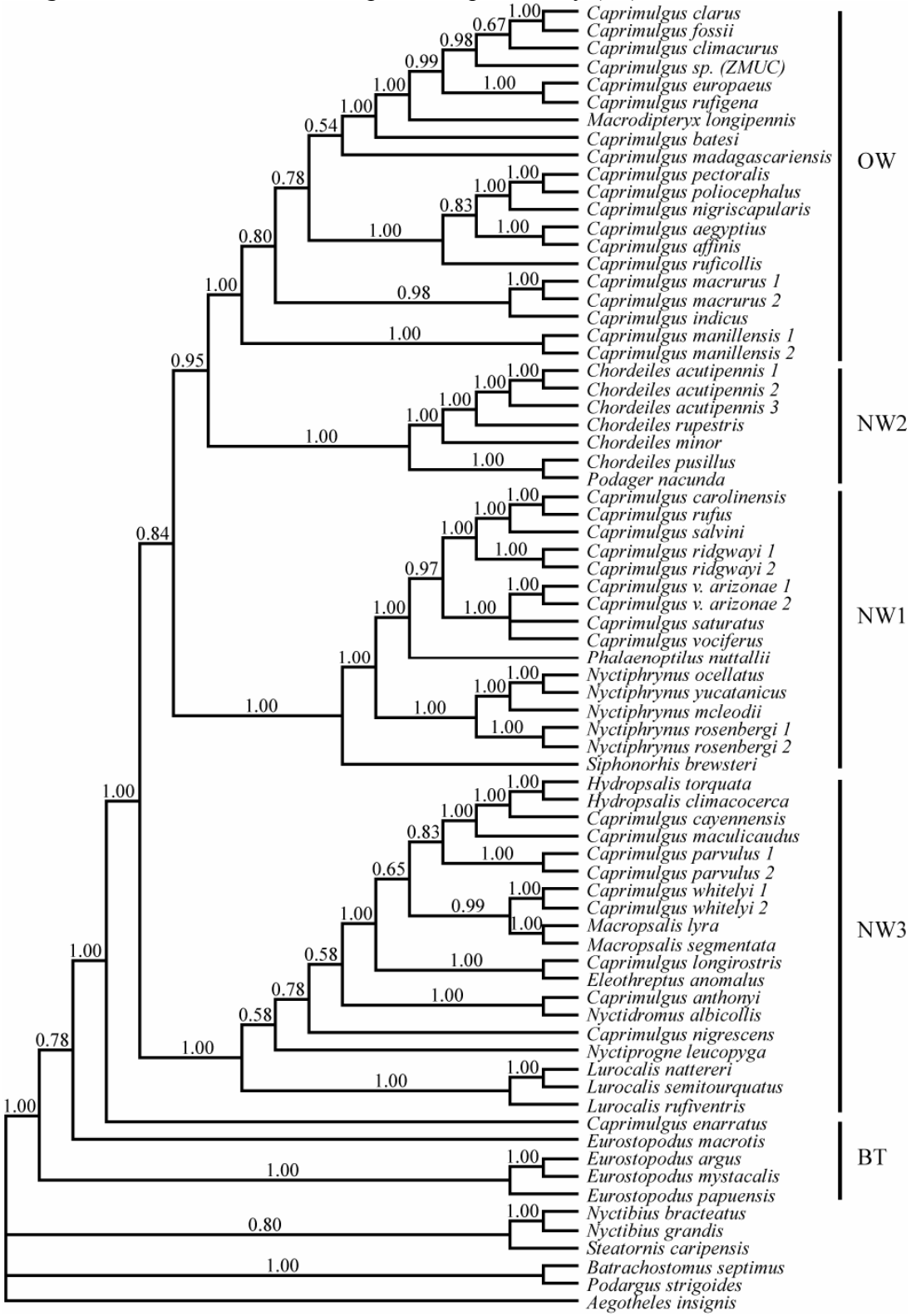
Appendix C: MP and MB trees for all genes combined

1. All genes MP. Strict consensus of one tree. Length = 6319; CI = 0.343; RI = 0.563. Numbers indicate bootstrap >50%.



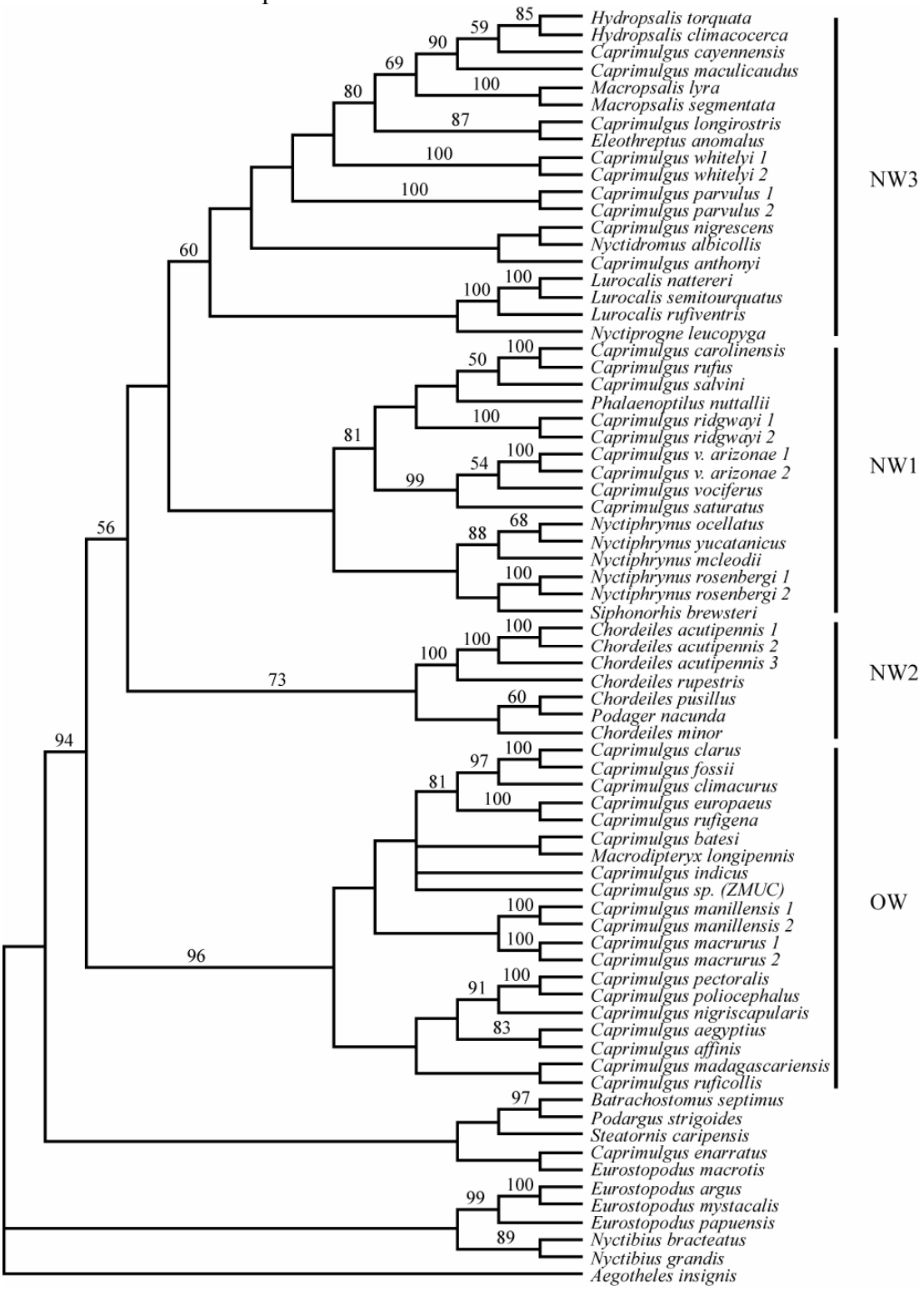
Appendix C: cont'd

2. *All genes MB.* Numbers indicate posterior probability (PP) >0.50.



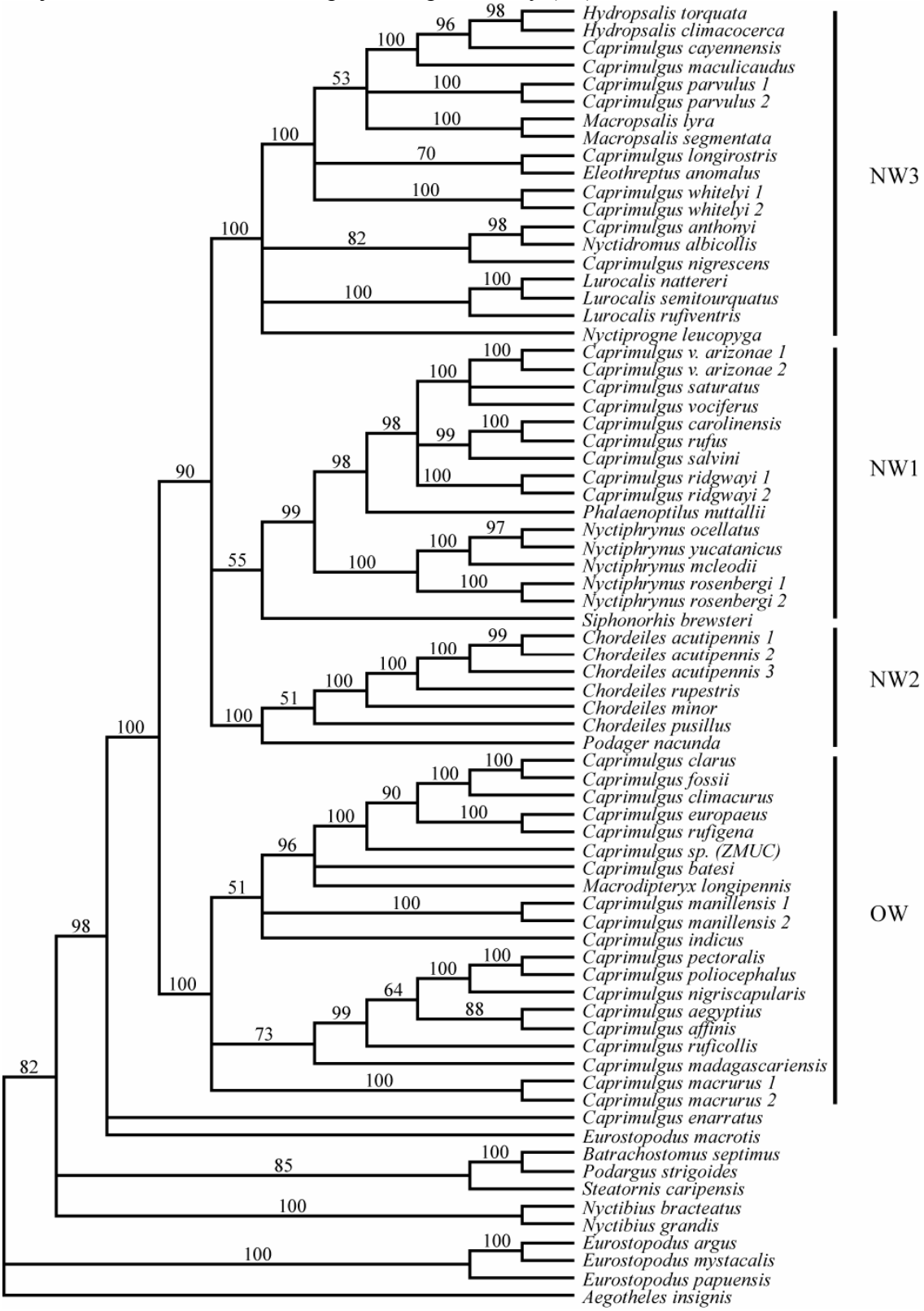
Appendix D: MP and MB trees for Cytb

1. Cytb MP. Strict consensus of two trees. Length = 4212; CI = 0.228; RI = 0.491. Numbers indicate bootstrap >50%.



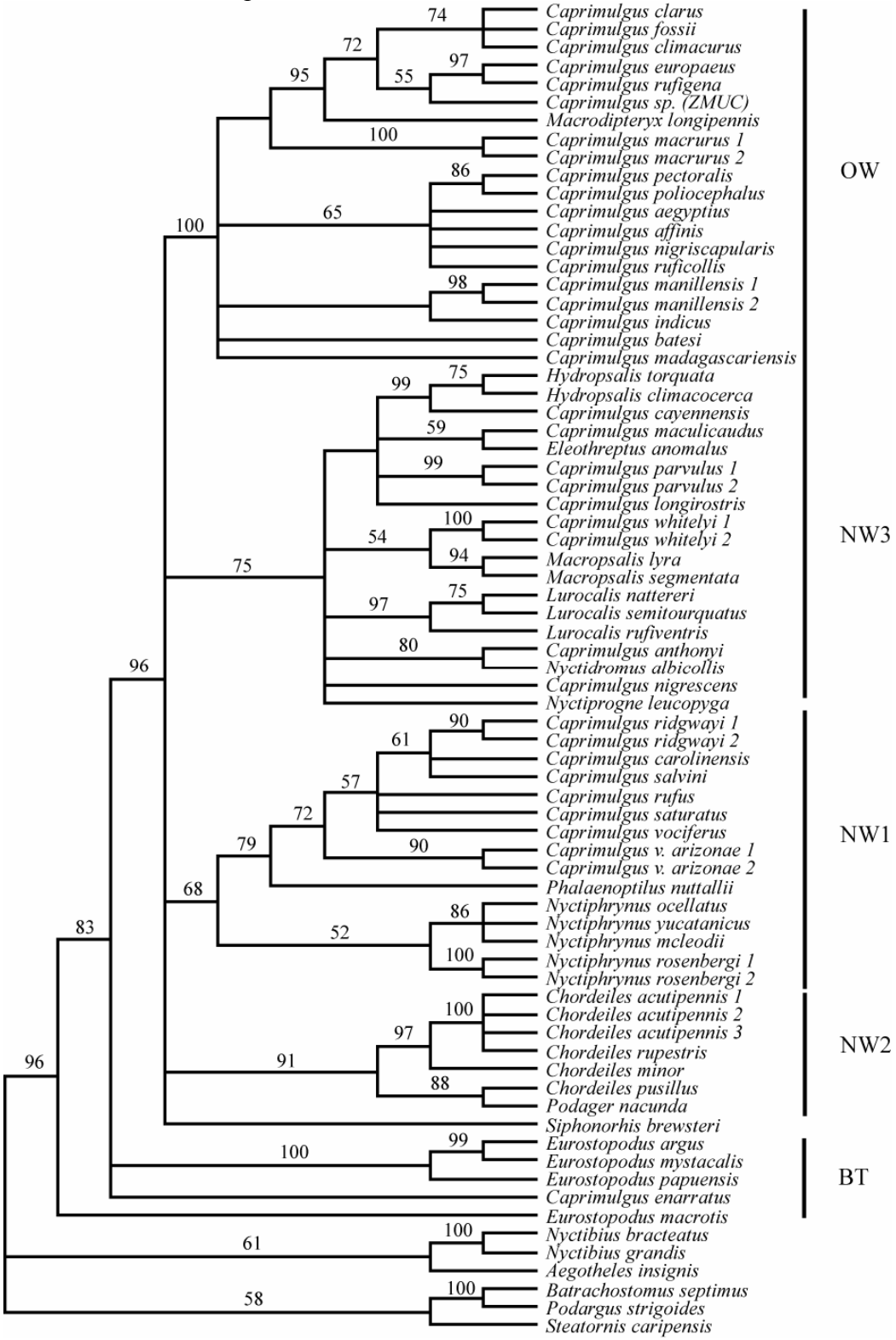
Appendix D cont'd

2. Cytb MB. Numbers indicate posterior probability (PP) >0.50.



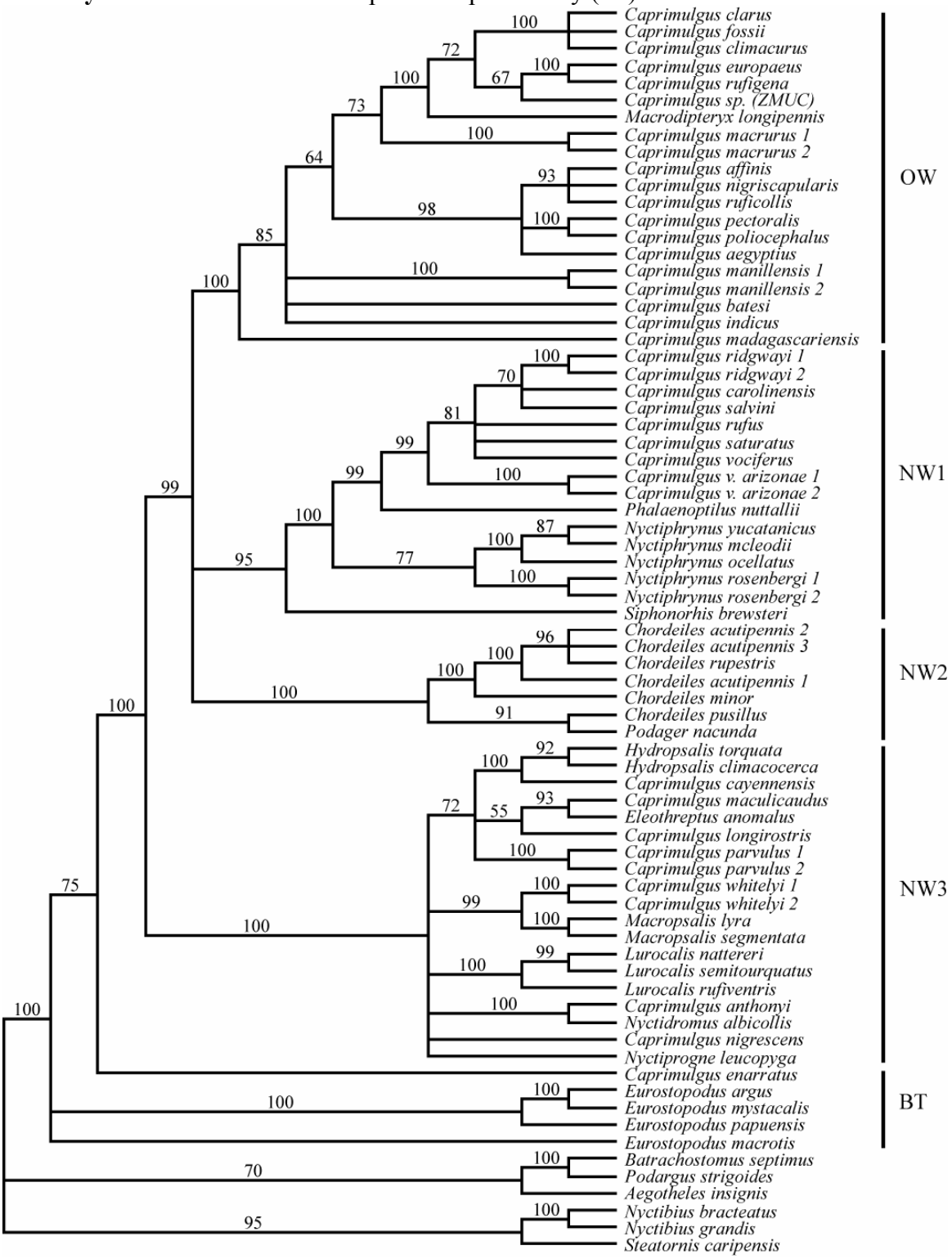
Appendix E: MP and MB trees for c-myc

1. C-myc MP. Strict consensus of 189,670 trees. Length = 692; CI = 0.572; RI = 0.753. Numbers indicate bootstrap >50%.



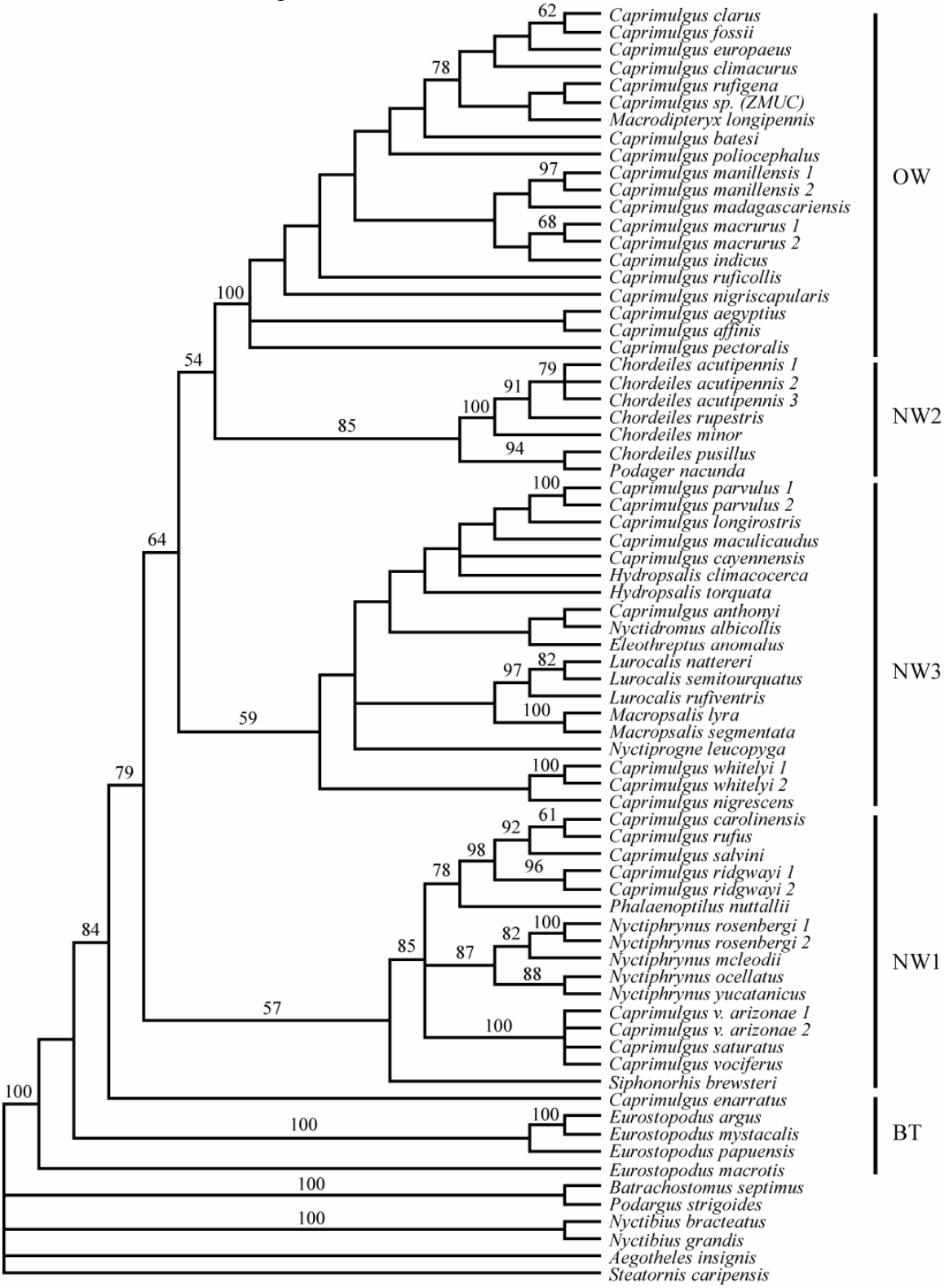
Appendix E: cont'd

2. C-myc MB. Numbers indicate posterior probability (PP) >0.50.

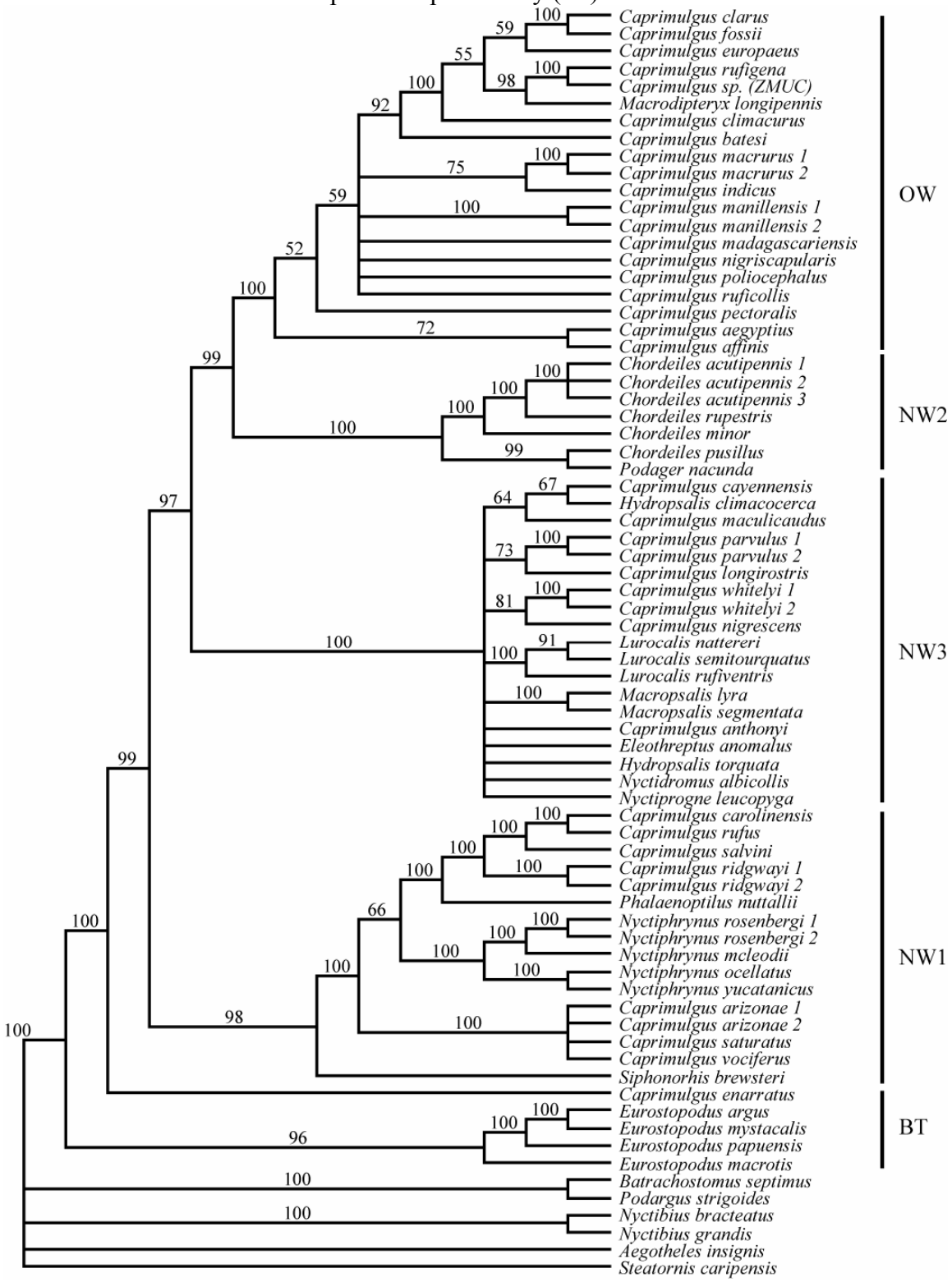


Appendix F: MP and MB trees for GH

1. GH MP. Strict consensus of 96 trees. Length = 1318; CI = 0.616; RI = 0.737. Numbers indicate bootstrap >50%.

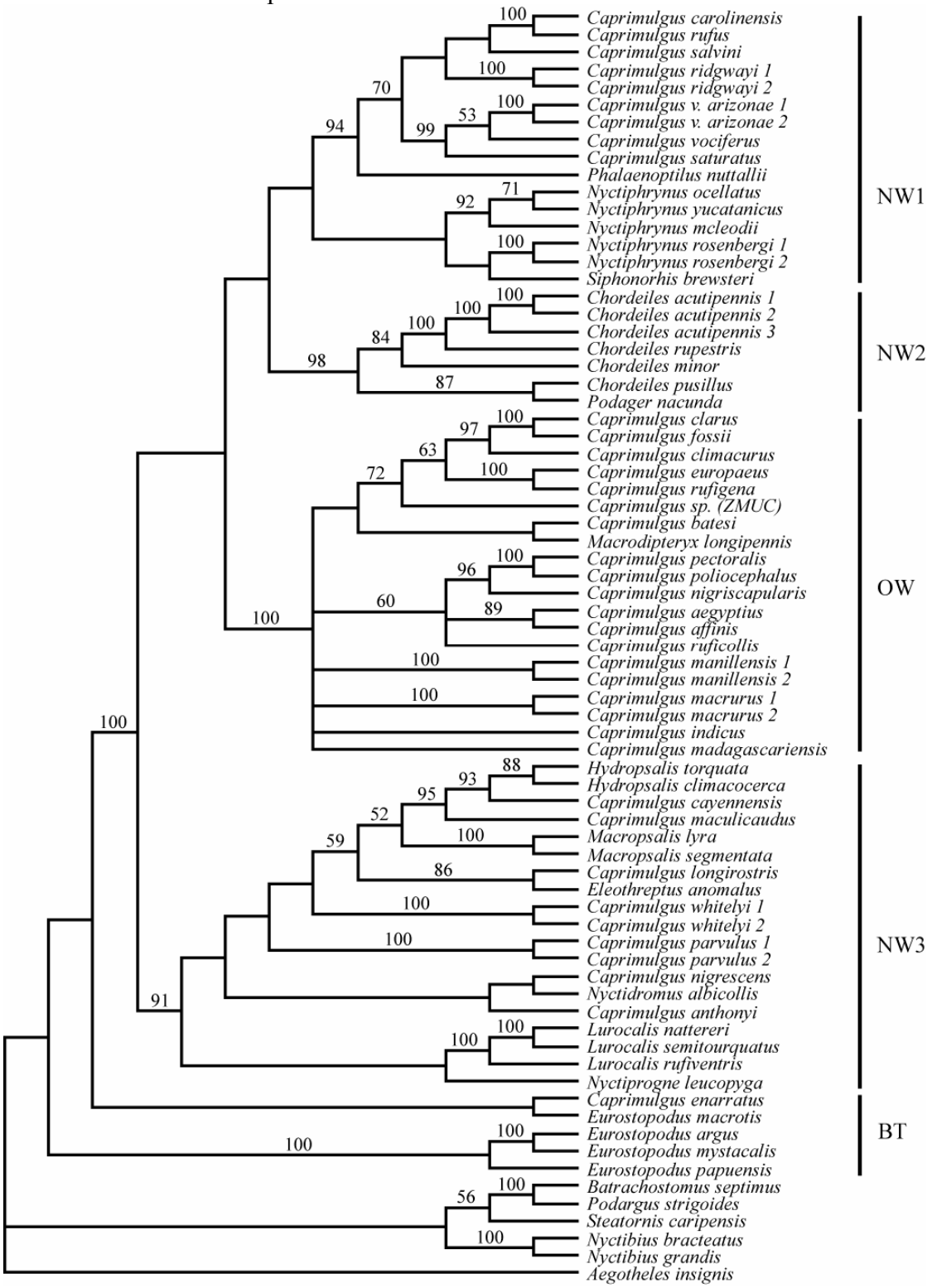


2. GH MB. Numbers indicate posterior probability (PP) >0.50.

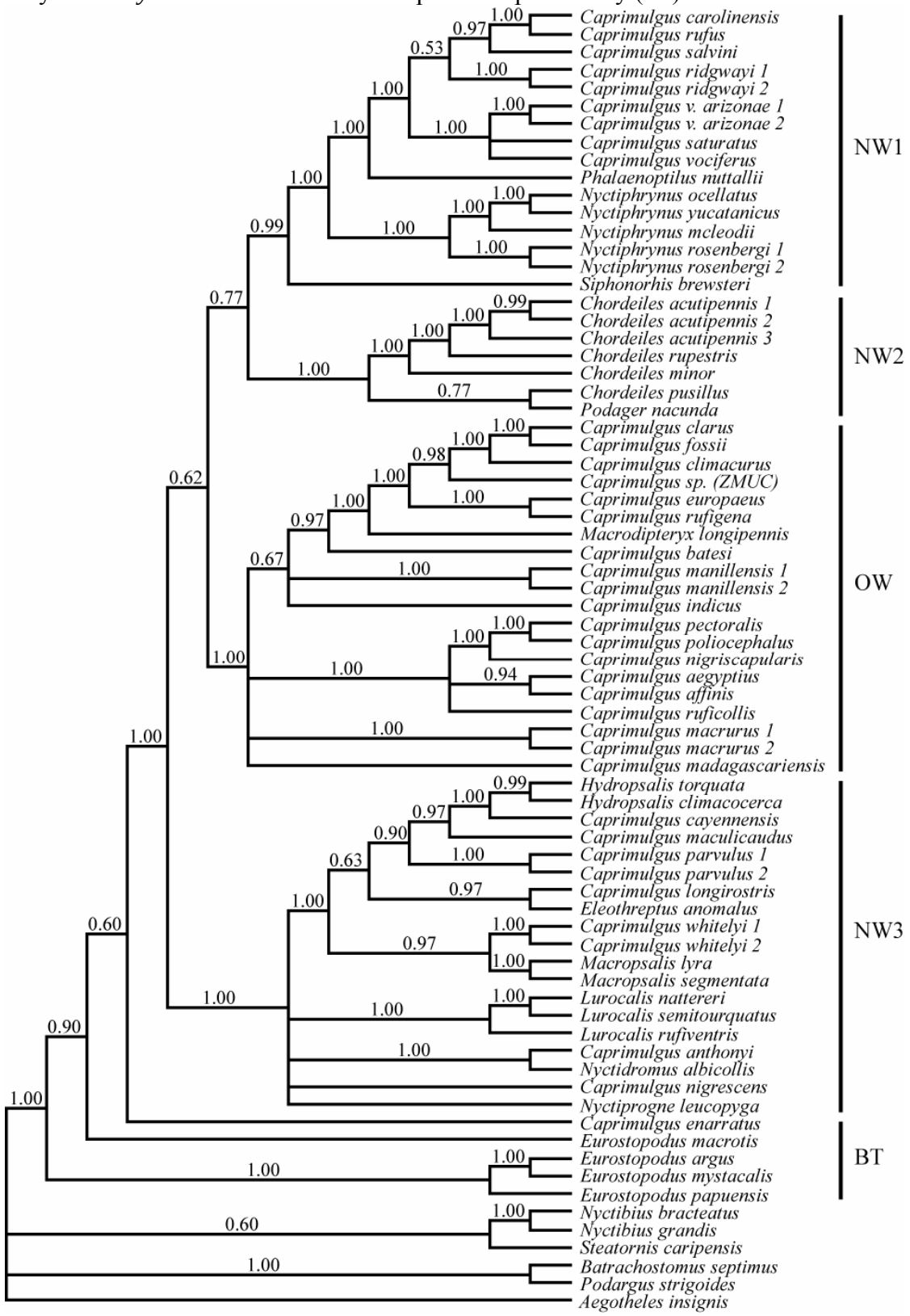


APPENDIX G: MP AND MB TREES FOR CYTB + C-MYC

1. Cytb + c-myc MP. Strict consensus of 5 trees. Length = 4948; CI = 0.274; RI = 0.527. Numbers indicate bootstrap >50%.



2. Cyt*b* + c-*myc* MB**.** Numbers indicate posterior probability (PP) >0.50.



APPENDIX H: NUCLEAR GENE ANALYSES

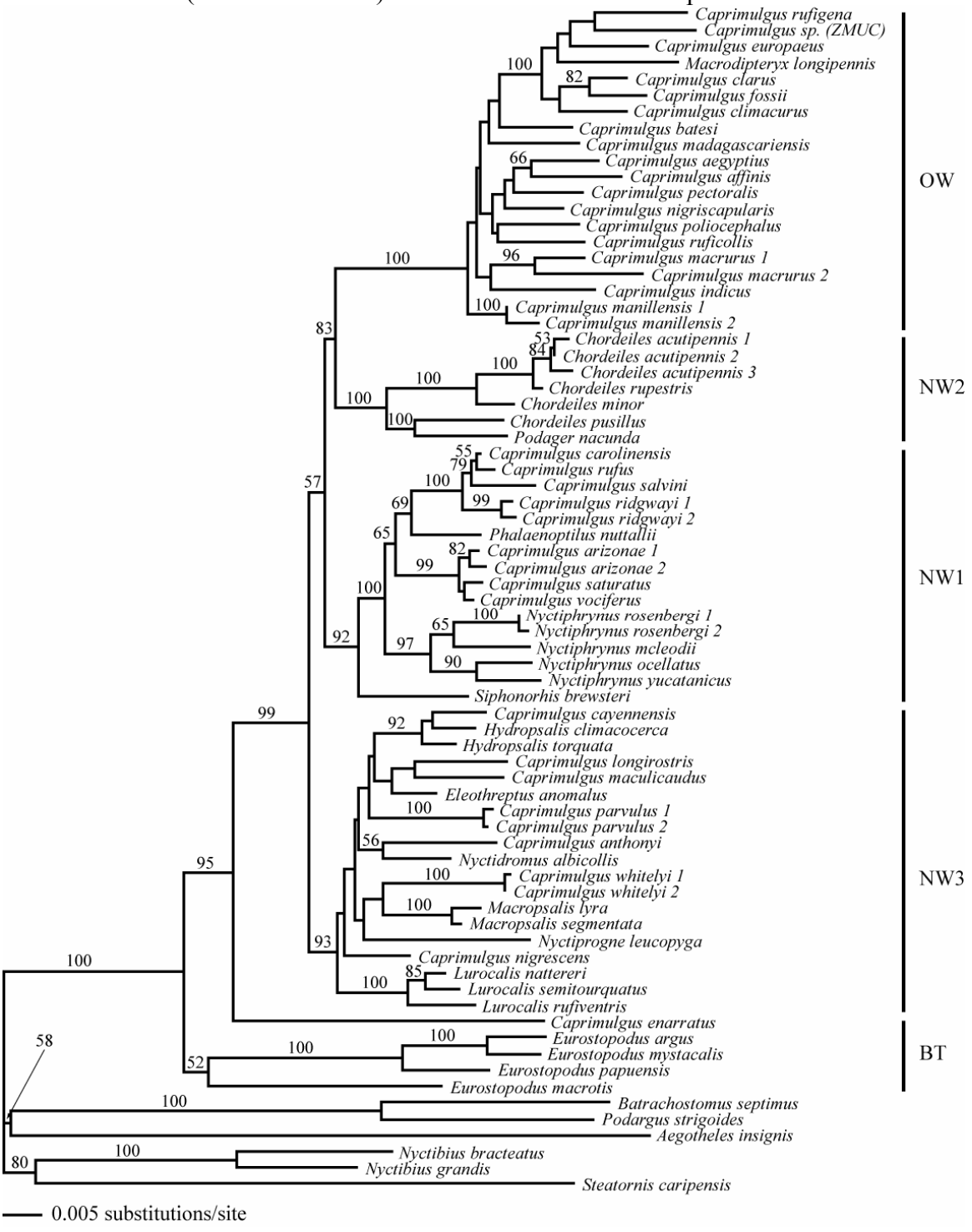
1. Phylogenetic methods

The two nuclear genes (c-*myc* and GH) were analyzed using parsimony, maximum likelihood and Bayesian inference methods. For parsimony analysis, 1000 random addition replicates and TBR branch swapping was initially performed with parsimony as the optimality criterion, saving only one of the best trees found (MULTREES=NO) per replicate. An additional search using TBR branch swapping on the saved trees was then performed saving all minimal trees (MULTREES=YES). To test the robustness of the tree, 100 bootstrap replicates were performed with 20 random sequence additions per pseudo-replicate. A limit of 100 trees (NCHUCK=100 CHUCKSCORE=1) was placed on the number of trees retained for swapping in each random addition replicate.

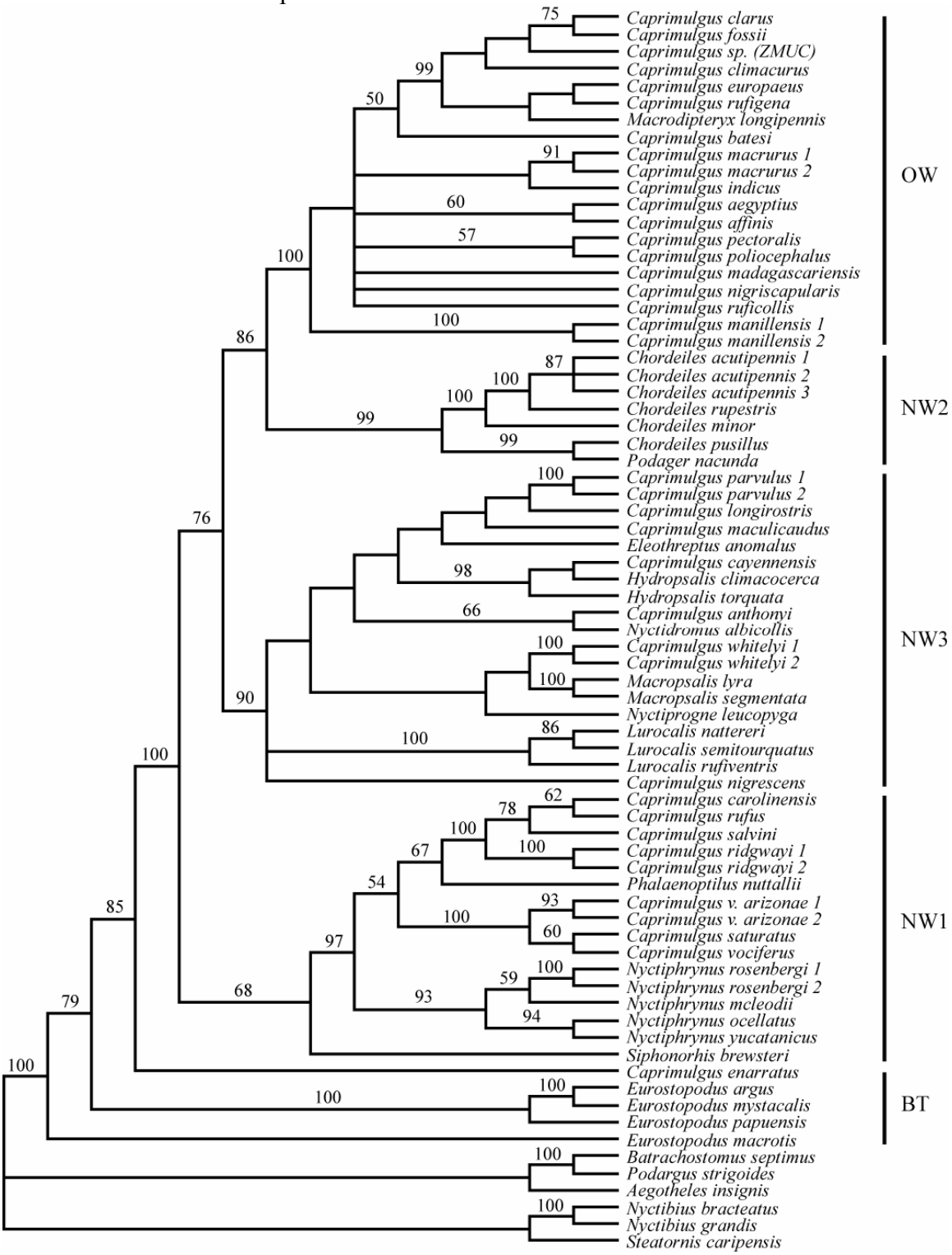
The model and parameters used for ML analysis was selected by Modeltest in a successive-approximations approach described earlier in Materials and Methods. The transversional model was used for analysis, with invariable sites and a gamma distribution (TVM + I + Γ) (see Table 3). A heuristic search was performed with 10 random sequence additions and TBR branch swapping using likelihood as the optimality criterion. Confidence in the hypothesized tree was estimated using 100 bootstrap replicates with one random sequence addition per bootstrap replicate.

MB analyses were performed using MrBayes. Four chains were run simultaneously for 10,000,000 generations and sampled every 500 generations. A GTR model was used for analysis. The dataset was analyzed with two partitions, one for each gene.

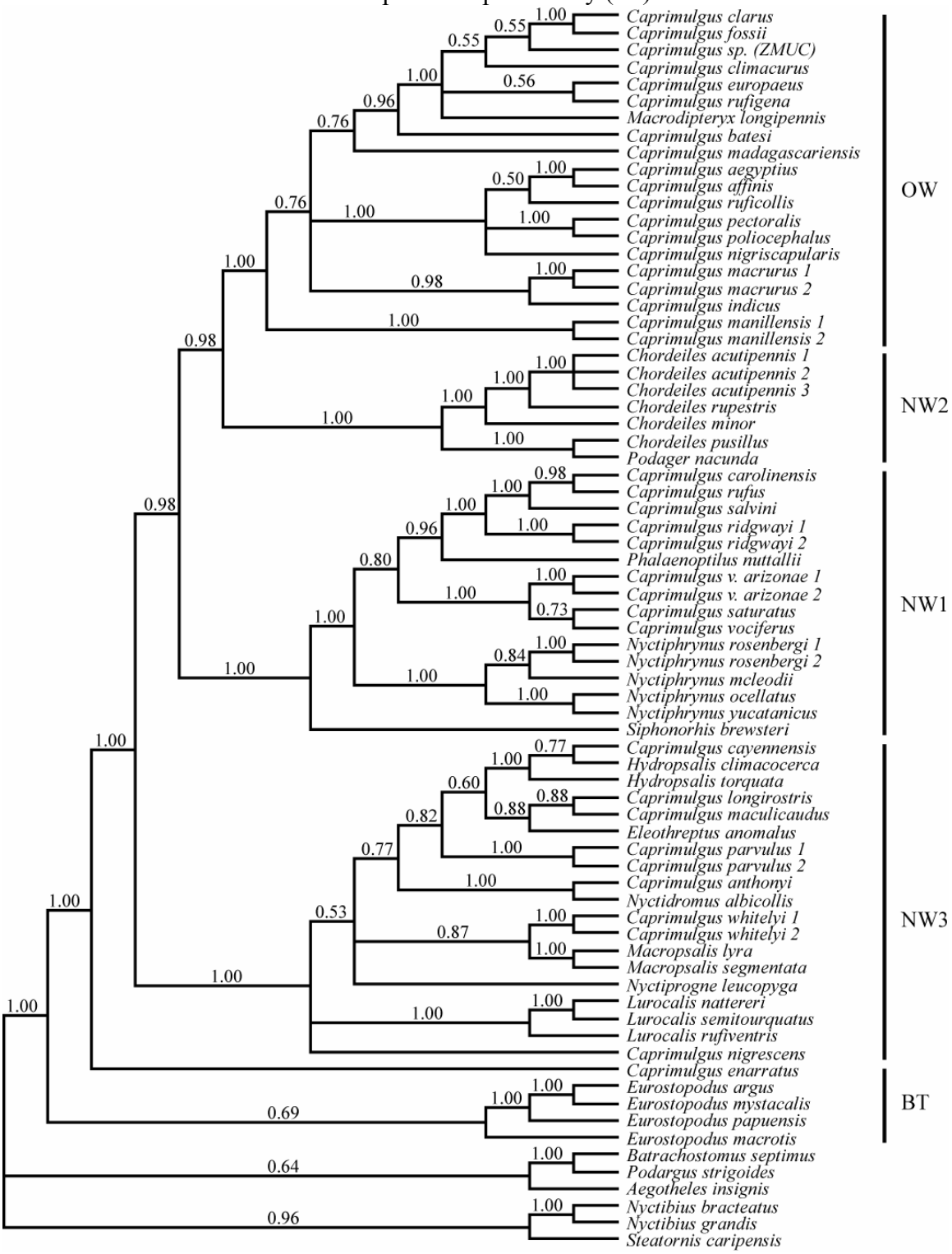
2. Nuclear ML. (-ln L = 16426.60). Numbers indicate bootstrap >50%.



3. Nuclear MP. Strict consensus of 60 trees. Length = 2045; CI = 0.591; RI = 0.732. Numbers indicate bootstrap >50%.



4. Nuclear MB. Numbers indicate posterior probability (PP) >0.50.



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